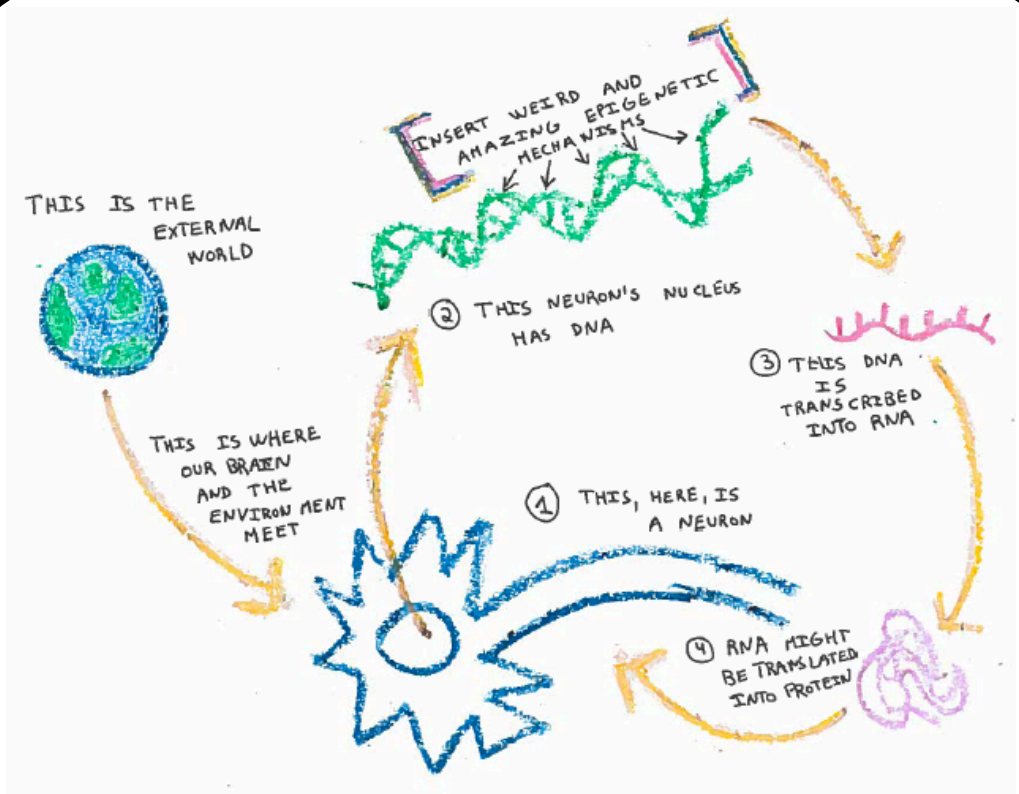
Behavioural and transcriptional plasticity in striatal circuits

From optogenetics to epigenetics

Pedro Nuno Galvão Ferreira



Dissertation presented to obtain the
Ph.D degree in Biochemistry – Neuroscience
Instituto de Tecnologia Química e Biológica António Xavier
Universidade Nova de Lisboa

Oeiras,
February, 2016

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA ANTÓNIO XAVIER /UNL
Knowledge Creation

Behavioural and transcriptional plasticity in striatal circuits

From optogenetics to epigenetics

Pedro Nuno Galvão Ferreira

Dissertation presented to obtain the
Ph.D degree in Biochemistry – Neuroscience

Instituto de Tecnologia Química e Biológica António Xavier
Universidade Nova de Lisboa

Research work coordinated by:

Fundação Champalimaud

FUNDAÇÃO CALOUSTE GULBENKIAN
Instituto Gulbenkian de Ciência

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Oeiras, February, 2016

INSTITUTO
DE TECNOLOGIA
QUÍMICA E BIOLÓGICA
ANTÓNIO XAVIER /UNL
Knowledge Creation

To my parents, who taught me how to be

(*Para os meus pais, que me ensinaram a ser*)

The work presented in this dissertation was carried out under the International Neuroscience Doctoral Programme (INDP, at the Champalimaud Neuroscience Programme, Champalimaud Centre for the Unknown) under the supervision of Dr Rui M. Costa, and the thesis committee supervision of Dr. Susana Lima and Dr. Carlos Ribeiro. Financial support was given by a doctoral fellowship from Fundação para a Ciência e Tecnologia (SFRH/BD/33279/2007, attributed to Pedro Nuno Galvão Ferreira), ERA-NET (F4T), European Research Council (COG 617142).

Did it matter then, she asked herself, walking towards Bond Street, did it matter that she must inevitably cease completely; all this must go on without her; did she resent it; or did it not become consoling to believe that death ended absolutely? but that somehow in the streets of London, on the ebb and flow of things, here, there, she survived, Peter survived, lived in each other, she being part, she was positive, of the trees at home; of the house there, ugly, rambling all to bits and pieces as it was; part of people she had never met; being laid out like a mist between the people she knew best, who lifted her on their branches as she had seen the trees lift the mist, but it spread ever so far, her life, herself.

Virginia Woolf

Mrs. Dalloway

Table of Contents

Page

Acknowledgements

It's been a long, and at times arduous, road. I've heard of someone who lived two thousand years ago who seems to have experienced a similar ordeal, the differences being that 1) He was left out to dry, 2) that in His case it ended with a heavenward flight three days after His final examination and 3) that He finished without a PhD. Too bad for Him. However, such as He taught, as well as the Buddha, "when life throws lemons at you, you turn them into papers as best you can" (I might be paraphrasing here), and the only way I was able to turn these sour citruses into anything resembling a decent scientific output was through the help of a bunch of very dedicated friends and colleagues. This group of scientists and muggles pushed me forward, provided coffee (and alcohol) when I needed it, technical support when it was required, and lent an ear whenever one was needed. They almost made me love people as much as I do books. To them I owe this thesis.

First and foremost of all, my supervisor. If this PhD has taught me anything, then it is the absolute and inescapable value of good mentoring. I owe Rui a debt of gratitude equivalent in mass to a black hole, the day a black hole ate a planet too many. He has been the Yoda to my Luke, the Gandalf to my Frodo, the Dumbledore to my Harry. This doctorate would not exist were it not for his enthusiasm and relentless belief in the value of one as clumsy as I. If anything, the existence of this very thesis is proof of his magical powers, both as a supervisor, a scientist and a wholehearted human being. He gave me freedom and support, always in the measure I needed, even if I didn't know it. If I am a scientist today, it is because of him. So thank you.

To my thesis committee, Susana Lima and Carlos Ribeiro, for the invaluable input and for keeping me on track. They taught me how smarts can meet scientific pragmatism. For that, as for everything else, thank you.

To my parents, *aos meus pais. Assim sendo, este vai em Português. Para além de terem-me dado biologicamente vida, os meus pais ensinaram-me a ser uma pessoa inteira, com todos os valores e idiosincrasias que isso acarreta. Eu não seria certamente a pessoa que hoje sou não fosse pelo constante apoio e carinho com o qual cresci, filho de pais que sempre quiseram que os filhos crescessem a ser tudo aquilo que conseguissem ser. Acima de tudo, por serem as pessoas mais completas que conheço, e por ensinaram-me que amar é mais que dizer – é viver e fazer. A eles esta tese é dedicada. (Como já mencionado umas páginas atrás, mas só para que não haja confusão, é mesmo para eles)*

To my sister, the Dee Dee to my Dexter, and a continuous source of inspiration. Besides having shared the insides of the same mammal – with a two-year interval – we share the same darkly humorous brain and an inner sense of how great books are. She is probably the only person I like more than books.

To Rui ("o Ribeiro," the cutest T. Rex), for putting up with me with the affection and patience of a saint, for showing me how to be both more human and a bit more generous, and for caring enough to teach me how to clean surfaces with three different types of detergent.

To the Costa lab, from the golden oldies of IGC days, to the new zygotes who are just beginning to appreciate how much they have struck gold here. A very very special thanks to Mafalda, my partner in crime, the Sam to my Frodo, without whom a part of this thesis would have had a much harder time coming to life (if at all).

To those special CNP people, team *mete-nojo* and extended family: Andreia, Mafalda, Grant, Ana (aka Belinha), João, Nicco, Susana, Anna, Libbi and Mai. If the end of the world ever came, I wouldn't want to spend it with anyone else.

To the initial PGCN (now INDP) 10: Rodrigo (especially for five years of amazing friendship and shared roof), Maria, Patrícia, Isabel, Margarida, Patrício, Mariana, Iris and Zé. It was a great ride.

To Lena, Sílvia and Daniela. Two of you are flying in from Stockholm and London for my PhD party (and the third would do so from Boston, were it not cheaper to fly in from Mars). I'll be the last one graduating, but I'm the cutest.

To the CNP, for creating this most amazing of creative atmospheres. I'm sure I'll never work in a place with a greater vibe, a superior summed IQ or better parties. You made me the scientist I hope I am today.

To the IGC, my first scientific home, and to its people. There I learned what doing great science meant, and to that I owe another eternal debt of gratitude. To Mike, whose mentoring helped me tread my first steps in science, and whose recommendation helped me get into this programme. To Rute, Ana Luísa and Sílvia, whose pragmatic spirit kept me on the right path.

To those geniuses, from Virginia Woolf to Fernando Pessoa and Sergei Prokofiev; from Giordano Bruno to Marie Curie, Albert Einstein and Barbara McClintock; from Marguerite Yourcenar to Eimear McBride and Martha Argerich. Of all of you I am in awe.

To Johannes Gutenberg, for inventing mechanical movable type printing. To you I owe my bankruptcy. My feelings for you resemble my intestines' towards warm chocolate: I love you and hate you.

This work was supported by Fundação Champalimaud, Fundação Gulbenkian, Fundação para a Ciência e Tecnologia (SFRH/BD/33279/2007), ERA-NET (F4T), European Research Council (COG 617142).

Resumo

O sistema nervoso central medeia a relação entre um organismo e o seu meio ambiente, construíndo uma ponte fisiológica moldada por uma série de estímulos que poderá envolver processos celulares e moleculares de diferentes ordens e diferente natureza. Esta tese explora plasticidade ao nível dos circuitos, bem como a nível molecular.

Na primeira parte desta tese, utilizando uma abordagem optogenética, exploramos o papel das duas principais vias de projecção dos gânglios da base no reforço de acções, mostrando que, ao contrário daquilo que foi previamente descrito, a via directa e indirecta suportam o reforço positivo de acções, mas reforçam estratégias de acção diferentes. Estes resultados mudam o conhecimento da fisiologia e função dos gânglios da base, e mostram que as vias directa e indirecta possuem papeis diferentes mas complementares na modulação das nossas acções.

Na segunda parte desta tese, investigámos o impacto da aprendizagem no mecanismo conhecido por pausa da ARN Polimerase II (ARN Pol II), medindo como a aprendizagem de uma habilidade motora modula a dinâmica de fosforilação desta macromolécula. Mostrámos que a aprendizagem motora impacta a fosforilação *in vivo* da subunidade RPB1 da ARN Polimerase II, e que esta modulação ocorre em genes de activação imediata (*immediate early genes*). Estes resultados fornecem uma nova demonstração de plasticidade ao nível da transcrição, demonstrando, pela primeira vez, que aprendizagem modula a pausa da ARN Polimerase II em genes no cérebro adulto.

Abstract

The central nervous system mediates the relationship between an organism and the external and internal worlds, building a physiologic bridge that is shaped by a plethora of stimuli and may involve cellular and molecular processes of different orders and diverse nature. This thesis explores plasticity both at the circuit and molecular levels.

In the first part, using an optogenetic approach, we explore the role of the two main striatal output pathways in action reinforcement, showing that, unlike what has been previously described, the striatonigral and striatopallidal pathways both support reinforcement, but of different action strategies. These results introduce new insight into our knowledge of basal ganglia circuit function, and demonstrate the concomitant but complementary role of the direct and indirect pathways in shaping our actions.

In the second part of this thesis, we investigate the impact of learning on the mechanism known as RNA Polymerase II (RNA Pol II) pausing, by analyzing how learning a motor skill modulates the striatal phosphorylation dynamics of this macromolecule. We show that indeed learning a skill impacts on the *in vivo* phosphorylation of the RNA Pol II RPB1 subunit carboxy terminal domain, and that this modulation occurs at immediate early genes. These results provide a new demonstration of plasticity at the transcriptional level, demonstrating, for the first time, that learning modulates RNA Pol II pausing in the adult behaving brain.

Half way along the road we have to go,
I found myself obscured in a great forest,
Bewildered, and I knew I had lost the way.

It is hard to say just what the forest was like,
How wild and rough it was, how overpowering;
Even to remember it makes me afraid.

So bitter it is, death itself is hardly more so;
Yet there was good there, and to make it clear
I will speak of other things that I perceived.

Dante Alighieri
The Divine Comedy – Inferno, Canto I

Chapter I

General introduction:

From behavioural to transcriptional plasticity

It is universally acknowledged that cells respond to different orders of stimuli, both internal and external, and modulate their gene expression programmes accordingly. As a cell type whose basic *modus operandi* consists in receiving information from its interconnected partners, integrating that information and mounting an appropriate response — be it producing an activity-induced action potential or shaping its very connectivity via dendritic spine remodeling — the neuron sits as the ideal laboratory for the study of activity-dependent gene expression. Ran and interpreted through a dynamically changing nervous system, experience molds anatomy and physiology.

The *Oxford English Dictionary* defines learning as "[an] acquisition of knowledge or skills through study, experience, or being taught." If thought of as a translation of performance into neuronal coding, learning can be drawn as a dynamic and functional link between behavioural and neuronal plasticity (Faulk and Dolinoy, 2011; Fischer, 2014). In an anatomically complex brain, this junction may be studied in many different systems, one of them being the array of interconnected nuclei known as the basal ganglia. The striatum sits as the primary gateway into the basal ganglia, doubling its complexity into two main output pathways, distinguishable mainly by their gene expression profiles and projections to different structures (reviewed in Kreitzer and Malenka, 2008). The so-called "direct" and "indirect" pathways (also known as, respectively, "striatonigral" and "striatopallidal" pathways) have traditionally had differential roles assigned to them along the skill learning curve, with the direct pathway responsible for the initial phases of learning, and the indirect pathway involved in the later phases of skill learning, when the memory of how to do something is consolidated (Gerfen and Young, 1988; Albin et al., 1989; Alexander and Crutcher, 1990; DeLong, 1990; Gerfen et al., 1990; Le Moine et al., 1991; Bernard et al., 1992; Mink, 1996; Ince et al., 1997).

As anatomically complex as the basal ganglia may be, this complexity is perfectly mirrored in its function, which spans the regulation of motor behaviour to calibrating the motivation-related valency of action performance (reviewed in

Graybiel and Grafton, 2015). However, there has been considerable controversy with mapping the roles of the direct and indirect pathways to motor and reward behaviours. It is a partial aim of this thesis, then, to elucidate the involvement of the direct and indirect striatal output pathways in one of these behavioural features: positive reinforcement.

As mentioned above, striatonigral and striatopallidal neurons are morphologically similar and need, subsequently, to be genetically identified. Given that a study which tries to link neuronal to transcriptional plasticity would be extremely enriched by the examination of these mechanisms with cell type specificity — especially when the circuits under scrutiny have such opposing functions — in this thesis we took advantage of the genetical identifiability of these two groups of neurons to attempt both activity manipulation and extraction of pure populations for both pathways.

As is often said, "you never forget how to ride a bike," for, once consolidated, motor skills can last a lifetime. As with many other types of learning, proper and long-lasting consolidation of a motor skill very likely requires adjustments to the way the genomes of the neuronal circuits supporting that learning are read. This being said, the need arises, not only to identify and capture the specific neurons underlying a learning process, but also the regions of the genome being dynamically read.

Behind the apparent simplicity of the cellular nucleus and its three billion base pairs (bp) or readily readable nucleotidic information, the mammalian genome presents an awesome challenge of molecular interpretation. Similar to opening the correct section on a two million-page book, reaching the specific nucleotide sequence embedded in a dynamic bundle of chromatin is a task of gargantuan precision. The multi-dimensional chromatin structures that contain and comprise the genome need not only to be decompacted and the underlying DNA sequence exposed, but the correct transcription machinery needs to reach and adequately bind to that specific site (reviewed in Hager et al., 2009; Levine et al., 2014). A multitude of molecular mechanisms — such as acetylation, ubiquitination or

methylation of the histone macromolecules of nucleosomes, or methylation of cytosines within DNA CpG dinucleotides — facilitate or hinder gene expression, in most cases by changing the biophysical relationships between DNA and the protein content of chromatin (reviewed in Wolf and Linden, 2012; Meaney and Ferguson-Smith, 2010). Once chromatin is open, scaffolding elements and transcription factors prime DNA for transcription, facilitating the binding of effector molecular complexes such as RNA Polymerase II (RNA Pol II) (reviewed in Hager et al., 2009; Levine et al., 2014). The entire process of transcription, including its prior and posterior events, can be regulated at different stages, one of them being the very progression of RNA Pol II throughout the transcription cycle. Initially discovered in *Drosophila melanogaster* during the 1970s and 1980s, RNA Pol II promoter-proximal "pausing" has emerged as a major player in transcriptional regulation at several levels of mammalian biology, from embryonic development to brain function (reviewed in Jonkers and Lis, 2015). Although a specific involvement of RNA Pol II pausing in brain physiology has been implied through the work of the Dudek lab (including a new taxonomical approach to "immediate early genes" [IEGs] based on their activity-dependent transcriptional dynamics) (Saha et al., 2011), no role has of yet been demonstrated for this mechanism in the adult, *in vivo*, brain in the context of learning.

As a biological mechanism that so intuitively links upstream cues and stimuli to transcriptional plasticity, RNA Pol II pausing sits as an ideal candidate to bridge dynamic neuronal activity — necessary for learning — to a flexibly read genome. With this in mind, we set out to explore the impact of learning a motor skill on RNA Pol II pausing in the mouse striatum. After training in a fast lever-pressing task, an initial examination of the global phosphorylation dynamics of RNA Pol II was followed by profiling of the binding kinetics of these phospho-variants to the promoters and gene bodies of IEGs. Our experiments provide, to the best of our knowledge, the first demonstration of RNA Pol II phosphorylation modulation in the adult brain in the context of learning.

Given the dual nature of this thesis, the general introduction will be divided into two parts. In the first part of the introduction, we will focus on the basic neurobiology of the basal ganglia and the underlying complexity — and polemics — behind the physiology and function of its two main output pathways. In the second part of the introduction, we will shift our attention to the intersection between epigenetic mechanisms and neurobiology, exploring in detail the interplay between nuclear architecture, RNA Pol II pausing and neuronal activity-dependent epigenetic transcriptional regulation.

Part 1 — Basal ganglia neurobiology

1.1 The basal ganglia: tuning in on the striatum

Santiago Ramon y Cajal once said "*The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory.*" Sitting underneath the columnar complexity of the cortex, the group of interconnected nuclei known as the basal ganglia has lost many of its functional and anatomical mysteries throughout the years. Dating back to the 1660s work of Thomas Willis, which first identified and systematized different subcortical structures, the basal ganglia saw its intricate structure and nuclei dissected and acknowledged throughout the following centuries (Steiner and Tseng, 2010). In 1941, Cécile and Oskar Vogt proposed the distinction of striatum (named by Samuel Wilson in 1912 due to its striated appearance) into caudate nucleus, putamen and nucleus accumbens (NAc) (Steiner and Tseng, 2010): the caudate-putamen constituting the dorsal striatum (primarily involved in motor control and habit/skill learning), with the NAc corresponding to the ventral striatum (traditionally involved in motivation and reinforcement) (reviewed in Graybiel and Grafton, 2015). A master regulator of motor behaviour and of the reinforcement value of learned actions, the basal ganglia are part of a series of loops linking several cortical areas, via basal ganglia, to the thalamus and back to the cortex (Joel and Weiner, 1994; Parent, 1990).

The striatum sits as the main entry point to the basal ganglia, receiving glutamatergic excitatory inputs from cortex and thalamus which synapse onto two distinct classes of striatal medium spiny neurons (MSNs) (reviewed in Kreitzer and Malenka, 2008). These GABAergic MSNs compose approximately 95% of all striatal neurons, the remaining 5% comprising aspiny GABAergic neurons and cholinergic interneurons (Kawaguchi, 1995; Bolam et al., 2000). The striatum owes its uniqueness, in great part, to this complete lack of glutamatergic cells (Kawaguchi, 1995; Bolam et al., 2000).

According to the classical view of basal ganglia information flow, a balance of glutamatergic and dopaminergic neurotransmission at the level of its entry point, the striatum, splits into a twofold array of projections, as striatal MSNs connect to different downstream nuclei (reviewed in Kreitzer and Malenka, 2008). Activation of the so called "direct pathway," composed of MSNs expressing the dopamine D_1 (D_1R) and muscarinic M4 receptors (Chrm4) that project directly to basal ganglia output nuclei, leads to GABAergic inhibition of these structures: the substantia nigra pars reticulata (SNr) and internal — or medial — globus pallidus (GPi or GPm); in turn inhibition of the SNr (the neurons of which are also GABAergic) results in disinhibition of its thalamic downstream glutamatergic targets and its excitatory transmission to the cortex (Gerfen and Young, 1988; Gerfen et al., 1990; Le Moine et al., 1991; Bernard et al., 1992; Ince et al., 1997). This direct connection of striatum to SNr gave the direct pathway its other name: striatonigral pathway (Albin et al., 1989; Alexander and Crutcher, 1990; DeLong, 1990). Inversely, activation of the "indirect pathway," comprising MSNs expressing the dopamine D_2 (D_2R) and adenosine A_{2A} receptors, indirectly projects to the SNr via the external — or lateral — globus pallidus (GPe or GPl) and subthalamic nucleus (STN); this inhibition of GABAergic GPe neurons leads to a disinhibition STN glutamatergic neurons, which in its turn activates the SNr-thalamus GABAergic neurons (Gerfen and Young, 1988; Gerfen et al., 1990; Schiffmann et al., 1991). The bypass, via GPe, of the striatum-SNr indirect pathway resulted in it being named striatopallidal pathway (Albin et al., 1989; Alexander and Crutcher, 1990; DeLong, 1990). The divergence in naming the striatonigral and striatopallidal as the direct and indirect pathways, respectively, hence stems from the direct pathway reaching SNr directly, while the indirect pathway bypasses it.

As mentioned above, these MSNs are divided into two separate populations — the direct and indirect pathways — based mainly on their genetic identities and subsequent protein expression for, morphologically, they are indistinguishable (Gerfen and Young, 1988; Gerfen et al., 1990; Le Moine et al.,

1991; Bernard et al., 1992; Ince et al., 1997). Each of the differentially expressed dopamine receptors (D_1R and D_2R) triggers distinct intracellular signaling pathways based on the G proteins it's linked to (reviewed in Calabresi et al., 2014).

The above biochemical differences, as well as the nuclei to which each pathway projects, further fueled the direct/indirect pathway dichotomy. The impact this view of pathway divergence had on behavioural function will be the focus of the next section.

1.2 A tale of twos: the roles in reinforcement and motor behaviour of the striatonigral and striatopallidal pathways, and the dorsomedial and dorsolateral striata

A selection of optimal actions amongst alternatives is essential to the way an organism relates to, and interacts with, an ever-changing world. This ability to learn and act according to experience requires a system that can encode action-outcome associations, be plastic enough to adapt behaviour according to dynamic changes in value, and initiate specific actions while inhibiting non-selected ones. The striatum sits at this functional juncture as also evidenced by its anatomical connectivity, as it receives inputs from varied cortical and limbic brain regions (reviewed in Ena et al., 2011).

Historically, basal ganglia function has been based on the dichotomic nature of its two main striatal output pathways, onto which an information processing duality is mapped (Gerfen and Young, 1988; Gerfen et al., 1990; Le Moine et al., 1991; Bernard et al., 1992; Mink, 1996; Ince et al., 1997). This influential view sees the direct pathway as facilitating or selecting appropriate motor sequences, while the indirect pathway inhibits or hinders movement (Albin et al., 1989; DeLong, 1990; Mink, 1996; reviewed in Kreitzer and Malenka, 2008). This dichotomy also extends to the different regions of the dorsal striatum, the dorsomedial and dorsolateral striata (DMS and DLS, respectively), with the first involved in goal-directed behaviours and the latter in more habitual behavioural

strategies (a division supported by further anatomical data, which shows afferent projections from frontal and parietal cortical areas to DMS and sensorimotor areas projecting preferentially to DLS) (reviewed in Hilário and Costa, 2008). The orbitofrontal cortex (OFC) has recently been shown, together with the DMS, as being engaged in goal-directed actions, with the same DMS/OFC neurons that support this action decreasing in activity; this is concomitant with DLS neuron activity increase as the animal shifts from a goal-directed to a habitual action (Gremel and Costa, 2013). In 2009, Yin et al. demonstrated a differential involvement of the dorsal striatum during the different stages of skill learning, with DMS more engaged in the initial acquisition of a skill and DLS starting to be involved early and continuing to be involved in late consolidation. It has also been shown that extensive training correlated with long-lasting changes in plasticity in both striatonigral and striatopallidal neurons in DLS, supporting the hypothesis that skill consolidation is dependent also on potentiation of indirect pathway neurons (Yin et al., 2009). This separation of functions becomes more complicated when one tries to fit together the direct/indirect pathway and DMS/DLS dichotomies, as both the DMS and DLS possess D_1R- and D_2R-expressing MSNs (D_1 and D_2 MSNs), resulting in an intermingling of direct and indirect pathway neurons throughout the dorsal striatum (reviewed in Ena et al., 2011).

As mentioned above, the direct and indirect pathways are hypothesized to exert opposing effects on motor behavior (Albin et al., 1989; DeLong, 1990; Mink, 1996; reviewed in Kreitzer and Malenka, 2008). This differentiation in action coding is supported by the idiosyncrasies in connectivity within each pathway, as both converge on the SNr and subsequently influence thalamic activity, with, however, the indirect pathway activating SNr-thalamus GABAergic neurons via STN glutamatergic disinhibition (Calabresi et al., 2014). The recent coming of age of optogenetics facilitated a more efficient and, for the first time, cell type-specific examination of the role of striatal output pathways in motor control (as well as in other behavioural settings). Cell type-specificity (as is the case of D_1 and D_2 MSNs) is a major issue in functional dissection of many neural circuits, as cell types are

often anatomically intermingled and thus extremely hard to tell apart if not through their genetic identity (Ena et al., 2011). This being said, traditional pharmacological and electrical stimulation techniques are insufficient for more precise, circuit-specific approaches. With the recent introduction, into mammalian neural cells, of single-gene component light-gated protein ion channels (most popularly, channelrhodopsin-2 [ChR2]), and subsequent millisecond-scale activation or inactivation of genetically-defined neuronal cell-types, scientists obtained unprecedented resolution for both the "where" and "when" of *in vivo* neuronal activity manipulation and baptized this approach "Optogenetics" (Boyden et al., 2005; reviewed in Yizhar et al., 2011).

In 2010, Kravitz et al. elaborated on the dual role of the direct and indirect pathways in motor control by expressing a Cre recombinase-dependent version of ChR2 in the DMS of D_1 and D_2 Cre-expressing MSNs (D_1-Cre and D_2-Cre mice, respectively), observing an increase in motor activity with D_1 stimulation and decrease when stimulating D_2 MSNs. The picture is more complicated, though, for concurrent direct and indirect pathway activation is observed preceding action initiation and termination (Cui et al., 2013). Simultaneous operation of direct and indirect pathway neurons may be key to integrate all the necessary inputs for a proper and functional motor response.

As hinted above, a go/no-go functional understanding of basal ganglia circuits, as it relates to motor behaviour, is entirely too simplistic (Calabresi et al., 2014). Recent work enhances this view by awarding to corticostriatal circuits a wider role in action initiation, performance and termination. This results in a much more complex view of sequence-related activity processing in the direct and indirect pathways, in which movement units are chunked into action sequences, with D_1 MSNs preferentially displaying continuous or sustained sequence-related activity and D_2 MSNs decreased or inhibited activity during sequence performance (Jin et al., 2014).

As we've seen, the striatal output pathways possess quite an integrative role in action performance: the "when" and "how" an animal does something. However, these basal ganglia subcircuits play an additional, very important role in the selection of action strategies, the "why" we perform a certain action — that is, the relationship that exists between an action and its outcome (reviewed in Macpherson et al., 2014).

Reinforcement learning — learning by trial and error within a contingency between and action and an outcome (possessor of a certain expected value) — may be defined as learning by interacting with an environment, and is at the very basis or instrumental conditioning (the learning of a certain action — or set of actions — that results in obtaining a reward and/or avoid punishment) (Yin and Knowlton, 2006; Macpherson et al., 2014). The performance of actions may then be dependent on an action-outcome (A-O) association, with action execution sensitive to changes in outcome value. In these cases, the action is dubbed goal-directed (Yin and Knowlton, 2006; Balleine et al., 2009). However, if an action is repeated without substantial changes in outcome value, it may become a habit, with further performance insensitive to changes in action-outcome contingencies (interaction with a stimulus results in a set response [S-R]) (Yin and Knowlton, 2006; Balleine et al., 2009).

Circuits for striatal-dependent instrumental learning have been previously identified as mentioned above: DMS involvement in goal-directed behaviours and initial skill acquisition; and DLS involved in habit formation and late skill consolidation (Yin et al., 2009; reviewed in Graybiel and Grafton, 2015). These long-lasting changes in DLS task-related neural activity have been shown to be pathway-specific, as they occur mainly in D_2 MSNs and are observed to a lesser extent in D_1 MSNs with prolonged skill training (Yin et al., 2009). Recently, it has been shown that striatal-specific deletion of $A_{2A}R$ (which colocalizes with D_2R) leads to deficits in habit learning (Yu et al., 2009). Animals may also shift between goal-directed and habitual action strategies, or generalize previously learned actions (instead or learning an action *de novo*) when faced with a novel challenge (Hilário et

al., 2012). Concomitantly, animals trained in a random interval schedule in an operant task (which biases behavior towards habits) show a similar rate of pressing for both a lever that has been associated with reward and a new, inactive lever. This generalization is abolished lesioning DLS (Hilário et al., 2012).

Actions may be performed in order to procure a stimulus — a resulting outcome — that may be rewarding, while avoiding other stimuli that are aversive. A positive and rewarding outcome for a performed action leads to a reinforcement of that specific action; with time and repetition, action performance may become less dependent on the previously learned reward value of that action and thus result in the formation of a habit. While D_1 neurons have ubiquitously been associated with a reinforcement role in action performance, D_2 neurons are usually implied in aversion learning. Optogenetic experiments have lent additional credibility to this claim, with D_1 stimulation inducing consistent reinforcement and D_2 stimulation resulting in aversion (Kravitz et al., 2012). Using a different approach for circuit-specific manipulation, Hikida et al. (2010) showed that inhibition of D_1 neurons decreases conditioned place preference previously associated with a reinforcing valence. Additionally, Durieux et al. (2009) demonstrated that D_2 neuron-specific ablation in the ventral striatum results in an increase in drug reinforcement (showing, similarly to Kravitz et al. [2010], an inhibitory role for striatopallidal neurons in motor activity with striatal-wide D_2 neuron ablation).

As we've seen, there have been extensive efforts towards functionally and anatomically characterizing the striatonigral and striatopallidal basal ganglia output pathways. This work has taken the basal ganglia field to a new understanding of how and why animals perform actions, and sheds new light on the role of the striatum in regulating motor behaviour and the reward value of an action. This will be further explored in chapter 2. Immense progress has also been made on the biochemical dissection of the direct and indirect pathways, studies that extend the idiosyncrasies of the striatonigral and striatopallidal pathways from function to molecules and their activity-dependent regulation. The examination of the

transcriptional output of genetically-defined neural circuits is a natural result of these new experimental approaches, as we will see in the next section.

1.3 New tools, new tales: striatonigral and striatopallidal molecular physiology and transcriptomics

A fuller understanding of genome sequences and function has taken the biomedical sciences to a fuller understanding of what a cell type is and how it can be biologically defined (Arendt, 2008; Deneris and Hobert, 2014; Trapnell, 2015). The development of bacterial artificial chromosome (BAC)-carrying transgenic mice, in which fluorescent protein genes are selectively expressed in specific neuronal subtypes under cell type-specific promoters, has allowed neuroscientists to functionally tackle neuronal circuits that would otherwise be extremely hard to individualize within the complex mammalian brain (reviewed in Durieux et al., 2011; Ena et al., 2011). Throughout the past decade and a half, the basal ganglia field has benefited enormously from these efforts that bridge molecular biology to systems neuroscience, and it has done so for two different experimental purposes: activity manipulation and visualization of neural circuits.

Recent D_1- and D_2-driven expression of Cre recombinase brought the Cre-lox system to basal ganglia research (Durieux et al., 2011; Ena et al., 2011). Some of the experimental consequences of this were the optogenetics studies mentioned above, which took cell type specificity to neuronal activity manipulation.

As suggested in the previous section, the striatonigral and striatopallidal pathways form an intermingled set of projections that can only be differentiated based on the specific set of molecules each cell type exclusively expresses (Ena et al., 2011). Given the molecular dichotomy between D_1R expression in striatonigral neurons and D_2R and A_{2A}R expression in striatopallidal cells, visualization of these two circuits was attained by targeting the expression of fluorescent reporter genes using the promoters for the above-mentioned receptors (Gong et al., 2003; Shuen et al., 2008). Besides confirming the *in vivo* functional divergence between the two

pathways, these studies opened new windows into the characterization of the striatonigral and striatopallidal circuits, for, in this context, visualization also means identification (Durieux et al., 2011). The creation of *Drd1a-dTomato* and *Drd2-EGFP* mice allowed for a finer *in vivo* probing of neurophysiological details for each pathway, but it also made something entirely different possible: the cellular isolation of pure striatonigral and striatopallidal populations for biochemical analysis (reviewed in Lobo, 2009). Following this experimental line, Lobo et al. (2006) applied fluorescence-activated cell sorting (FACS) and microarray analysis to the striata of *Chrm4-EGFP*, *Drd1a-EGFP* and *Drd2-EGFP* mice. Besides identifying a new set of differentially expressed genes between striatonigral and striatopallidal neurons, some of which with previously described clinical implications, this study provided an experimental framework for profiling cell type-specific gene expression dynamics.

Given the extreme and intricate cellular heterogeneity found in the mammalian brain, cell type-specific analyses of gene expression provide an essential improvement in biological resolution (Ena et al., 2011; Trapnell, 2015). These tools open interesting avenues for further scientific scrutiny. One possible and quite interesting next step involves the application of these novel cell type-specific isolation techniques, and subsequent gene expression analysis, to behaviorally relevant neuronal circuits. This will allow for a full bridging of neuronal and behaviour plasticity, taking the previously observed complexity in neural circuit function to the neuronal nucleus and it's dynamic expression.

During the preamble to this introduction, we mentioned the bike-riding metaphor of learning a long-lasting skill. We also examined what we define as "learning," and considered how the acquisition of the knowledge of "how to do something" is conveyed through experience and repetition. It is precisely to that directed shaping of our brains by experience, that acquisition and consolidation of "how to," mediated by functional changes within specific neuronal circuits, and the

indentation it leaves on their genomes (as we saw, the modulation of the way neuronal genomes are interpreted), that we turn to next.

Part 2 — Epigenetics in brain function

2.1 A small introduction to epigenetics

If one thinks of the history of biology — and that of genetics in particular — over the first half of the twentieth century, much of the struggle was concentrated on finding the chemical basis of heritability. The actual term "genetics" was only coined by William Bateson in 1905, quite after Gregor Mendel's 1850s and 1860s seminal experiments on the rules and patterns behind the inheritance of traits were rediscovedred (Krebs et al., 2014). The jump from so-called "Mendelian" or classical genetics to molecular genetics — that is, the search for the nature and regulation of the cellular molecules behind inheritance — was made possible by the 1911 and 1913 work of Thomas Morgan and Alfred Sturtevant on chromosomes and genetic linkage (Krebs et al., 2014). Between 1928 and 1952, with Griffith's discovery of bacterial transformation, the Avery-MacLeod-McCarty experiment (identifying deoxyribonucleic acid [DNA] as the chemical principle behind the phenomenon identified by Griffith) and the Hershey-Case experiment (demonstrating DNA, and not protein, as the genetic material mediating viral infection of bacteria), did molecular genetics come into full being (Krebs et al., 2014). On the 25th of April 1953, Watson and Crick published their seminal two-page paper proposing a chemical structure for DNA (using unpublished — and involuntarily supplied — DNA X-ray diffraction data obtained by Raymond Gosling and Rosalind Franklin) and its obvious impact on information transfer in living matter (Watson and Crick, 1953).

By 1942, though, C. H. Waddington was already coining the term "epigenetics" to describe the interaction between genes (regardless of their — at the time unknown — biochemical nature) and their environment to produce a phenotype (Waddington, 1942). This epigenetic environment, or "landscape," served as a useful metaphor for biological development, intuitively illustrating the way genetic information interacts with environmental cues (Waddington, 1942).

Waddington's epigenetic landscape illustrates this interaction during development and subsequent stepwise cell-fate decisions leading to differentiation into multiple cell types (Waddington, 1942). However, an epigenetic regulation of gene function does not — and indeed is not — only applicable to development, as much of current epigenetics research concerns itself with adult somatic cell function (reviewed in Faulk and Dolinoy, 2011; Wolf and Linden, 2012). This interaction between the genome of an adult, terminally differentiated cell and a set of intrinsic or extrinsic cues will now be discussed in more detail.

Ever since Alfred Sturtevant showed genes to be linearly distributed on chromosomes that scientists have wondered how this information is read (Sturtevant, 1913). In much the same way as we find and interpret a word in a book, in order for a gene to be "read" — i.e. transcribed — that section of the genome must be accessible to the necessary regulatory and transcriptional machinery (reviewed in Hager et al., 2009). If we understand transcription as a three-way process, then besides the "message" and the "reader" (that is, respectively, the gene and the transcription machinery, including the enzyme RNA Pol II), "markers" that keep the message available and readable — similarly to a finger keeping a book page open — must also be in place (Hager et al., 2009; Meaney and Ferguson-Smith, 2010). Consequently, the genome should be viewed as a three-dimensional structure, the accessibility of which is tightly regulated (reviewed in Chakalova and Fraser, 2012).

If we zoom in on a eukaryotic cell nucleus during interphase, we find a variably compacted mesh of chromatin, the macromolecular complex composed of DNA, protein and ribonucleic acid (RNA) that packages the genome and controls gene expression and replication (reviewed in Chakalova and Fraser, 2012). Either DNA or the protein complement of chromatin may be chemically modified to manipulate the accessibility of DNA within the chromatin fiber, often by modifying the electrostatic interactions between DNA and surrounding proteins (Wolf and Linden, 2012; Meaney and Ferguson-Smith, 2010). DNA methylation is the best-

described DNA modification that impacts gene expression, usually consisting in the addition of a methyl group to cytosines within CpG dinucleotides by DNA methyltransferases (DNMTs), resulting mainly in transcriptional repression (reviewed in Schübeler, 2015).

In eukaryotes, the basic unit of chromatin is formed by nucleosomes, macromolecular complexes composed of two sets of the four core histones (H2A, H2B, H3 and H4) and around 147 base pairs of DNA wrapped around the histone octamer (reviewed in Qureshi and Mehler, 2014; Hager et al., 2009). These histone proteins may be subject of several sets of post-translational modifications (PTMs), amongst which phosphorylation, glycosylation, ubiquitination, S-nitrosylation and methylation, but most famously N-terminal lysine acetylation (Meaney and Ferguson-Smith, 2010; Riccio, 2010). Histone acetylation by histone acetyltransferases (HATs) results in more relaxed and accessible chromatin, while removal of acetyl groups by histone deacetylases (HDACs) results in tighter and less accessible chromatin (Meaney and Ferguson-Smith, 2010; Riccio, 2010). Chromatin — and by extension DNA — accessibility, however, is only part of the transcriptional puzzle. The spatial organization and positioning of the eukaryotic genome within the nucleus is an extremely well regulated process, with some loci displaying dynamic relocation upon transcriptional activation (Chakalova and Fraser, 2012; Bickmore and van Steensel, 2013). A preferential interaction with euchromatic (transcriptionally active) or heterochromatic (transcriptionally inactive) nuclear regions may also be the partial result of histone PTMs and local chromatin structure, intimately connecting chromatin modifications and genome positioning (Chakalova and Fraser, 2012; Bickmore and van Steensel, 2013). As mentioned above, RNAs may also modulate gene expression at the level of chromatin (Hamby et al., 2008; Zaratiegui et al., 2007). Small interfering RNAs (siRNAs) have been shown to bind to DNA and enable siRNA-mediated gene silencing, also facilitating long-term gene silencing by recruiting HDACs and DNMTs and subsequently modulating chromatin structure (Zaratiegui et al., 2007).

In the next section we will introduce in more detail the fine interplay between chromatin dynamics and gene expression in the brain, and focus on the curious peculiarities of epigenetic regulation of neuronal activity-dependent gene expression.

2.2 Neuronal activity-dependent gene expression

Animals depend on the correct interpretation of external and internal cues into an appropriate behavioural output in order to survive, making the nervous system an anatomical and physiological relay station between an animal and the surrounding environment (reviewed in Wolf and Linden, 2012). This experience of — and interaction with — an environment is conveyed through changes in neuronal connectivity, structure and activity that mold neural circuits in an activity-dependent manner for short- or long-lasting changes (reviewed in West and Greenberg, 2011). This phenomenon of neuronal adaptability is known as "neuroplasticity" (reviewed in West and Greenberg, 2011; Lyons and West, 2011). With the increase in organismal and functional complexity, more and more intricate molecular mechanisms were selected by evolution as epigenetic adaptation systems, including the several layers of epigenetic regulation briefly mentioned in section 2.1 (Wolf and Linden, 2012). A good example of this environment-to-genes axis is the 2007 Fischer et al. study showing a direct connection between associative and spatial learning and chromatin remodeling (via environmental enrichment-induced hippocampal histone acetylation and methylation of histones H3 and H4).

Many of these mechanisms were built into the signaling cascades behind neuronal plasticity and learning/memory, hinting towards an intimate — and many times necessary — link between neuronal activity, epigenetic adaptation and the genome's output (Wolf and Linden, 2012; West and Greenberg, 2011; Lyons and West, 2011). Epigenetic regulation mediates neuroplasticity via a multitude of molecular interactions between chromatin remodeling enzymes (such as HDACs), Ca^{2+}-dependent signaling proteins and activity-dependent transcription factors

(Riccio, 2010; West and Greenberg, 2011; Lyons and West, 2011). The translation of an extracellular signal into an intracellular one (or second messenger) is dependent on the specific signal and receptor involved, with many of these receptor proteins coupled to intracellular second messenger systems that regulate, in their own term, the activity of effector enzymes and downstream target proteins (i.e. ion channels and transcription factors) (Lyons and West, 2011; Goldie and Cairns, 2012). The Ca^{2+} second messenger system is based on Ca^{2+}-sensitive proteins (such as Ca^{2+}/calmodulin-dependent protein kinases, CAMKs) phosphorylating downstream targets that modulate gene expression: CAMKII, for example, phosphorylates the cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), a transcription factor with a widely documented role in long-term memory formation and consolidation, resulting in the induction of specific gene expression programs; CREB may also be activated by the cAMP-dependent protein kinase (PKA) via a different second messenger system, involving a G-protein-coupled receptor (GPCR)-mediated intracellular increase in cAMP (Dash et al., 1990; Bourtchuladze et al., 1994; reviewed in Lyons and West, 2011). CREB then binds to cAMP response elements (CRE) DNA sequences, inducing the activity of many activity-regulated genes, such as the IEG *c-Fos* or the *brain-derived neurotrophic factor* (BDNF) (Barco and Marie, 2011; Lyons and West, 2011).

One of the binding partners of CREB, called CREB-binding protein (CBP), is involved in the transcriptional co-activation of several transcription factors, has inherent acetyltransferase activity of histone and non-histone proteins and locally remodels chromatin structure, as well as recruiting and stabilizing RNA Pol II (Barco and Marie, 2011; Lyons and West, 2011).

As mentioned above, *c-Fos* presents a paradigmatic example of activity-dependent gene expression (reviewed in Flavell and Greenberg, 2008). An IEG, *c-Fos* has been present in the neuroscientific toolbox for some time now as an activity marker, for *c-Fos* messenger RNA (mRNA) — and downstream protein — upregulation implies recent neuronal activity (Flavell and Greenberg, 2008; Saha

and Dudek, 2013). *c-Fos* was the first gene for which Ca^{2+}-dependent promoter-proximal *cis*-acting regulatory elements were identified, in this specific case located 100 base pairs upstream of the *c-Fos* transcriptional start site and named calcium response element (CaRE) (Montminy et al., 1986). The CaRE sequence is similar to CRE elements identified in other gene promoters (such as the *somatostatin* gene [Montminy et al., 1986]). A second regulatory element was identified within the *c-Fos* promoter, also identified as Ca^{2+}-dependent, termed serum response element (SRE) (Montminy et al., 1986; reviewed in Flavell and Greenberg, 2008). The activity-dependent transcriptional regulation of IEGs will be further explored in chapter 3.

As we've seen, a significant epigenetic layer is present and necessary for activity-dependent neuronal gene expression (reviewed in Puckett and Lubin, 2011). Many of these epigenetic events have been found — and were initially identified — outside the brain, but many do seem to be highly enriched in the nervous system (Lyons and West, 2011; Puckett and Lubin, 2011). The methyl CpG binding protein 2 (MeCP2), an additional subject of Ca^{2+}-dependent activation by phosphorylation, is present in high levels in mature neurons where it both represses and — more controversially — activates gene expression (Chahrour et al., 2008; reviewed in Guy et al., 2011). MeCP2 recruits HDACs to preferentially methylated DNA (5-methylcytosine [5mC]) sites and is present at near histone octamer level in neuronal nuclei (Nan et al., 1993; Chahrour et al., 2008; Skene et al., 2010; Mellén et al., 2012). 5mC may be converted to 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation (TET) enzymes, an epigenetic mark which is highly present in the brain, preferentially at active genes, where MeCP2 is the major 5hmC-binding protein (Mellén et al., 2012). Recently, Rudenko et al. (2013) showed that neuronal TET1 is necessary for the regulation of memory extinction and IEG expression, such as that of *c-Fos*, the activity-regulated cytoskeleton-associated protein (*Arc*) and the neuronal PAS domain protein 4 (*Npas4*) genes.

Many of these epigenetic mechanisms have also been found specifically in the basal ganglia. TET1 has been shown to be downregulated in mouse NAc as a result of cocaine administration (Feng et al., 2015). Still in motivation and reward learning, striatal CBP-mediated histone H4 acetylation at the *fosB* promoter (Levine et al., 2005), histone H3 acetylation at the *BDNF* promoter (Kumar et al., 2005) or CREB-dependent striatal microRNA metabolism (Hollander et al., 2010; Im et al., 2010) have all been linked to cocaine-induced plasticity, or altered spine plasticity in MeCP2-deficient mice due to amphetamine exposure (Deng et al., 2010). A specific chromatin PTM, phosphorylation of histone H3 on serine-10 (H3-Ser10P), has been widely studied in striatal neurons (reviewed in Matamales and Girault, 2011). H3-Ser10P is hypothesized as promoting chromatin decondensation and subsequent gene expression (Johansen and Johansen, 2006) and, when combined with H3 lysine-14 methylation, is associated with *c-Fos* hippocampal transcription in neurons (Crosio et al., 2003). H3-Ser10P has also been shown to occur specifically in striatonigral neurons after acute cocaine treatment (Bertran-Gonzalez et al., 2008).

Recently, a new set of *cis*-acting epigenetic regulatory mechanisms was identified in the brain. Kim et al. (2010) demonstrated activity-dependent CBP-binding to enhancers in mouse cortical neurons, resulting in RNA Pol II recruitment and transcription of a new class of enhancer RNAs (eRNAs), specifically at enhancers actively involved in mRNA synthesis (and marked by histone H3 monomethylation of lysine 4). Some of these activity-dependent enhancers have also been shown to require c-Fos binding (which was believe to bind mainly to promoters), suggesting that c-Fos (that, together with other IEGs, regulates the expression of late-response genes) also controls neuronal activity-dependent gene expression at the level of enhancers (Malik et al., 2014).

As we've seen, activity-regulated gene expression depends highly on the creation of a chromatin environment permissive for effective transcription (Lyons and West, 2011; Puckett and Lubin, 2011). In neurons, this massive coordination of molecular mechanisms links neuronal activity to the transcription of selected

groups of genes, and it may be regulated from the most upstream signaling cascades responding to ligand-mediated receptor activation or intracellular Ca^{2+} influx, to the facilitation or repression of chromatin binding by transcriptional effectors and regulators (Lyons and West, 2011; Puckett and Lubin, 2011). The behaviour of RNA Pol II and its phosphorylation dynamics along the transcription cycle is a story in its own right. That is the story of RNA Pol II pausing — one that will be told in the next section.

2.3 RNA Polymerase II phosphorylation dynamics: poised memories

In 2001, the first draft sequences of the entire human genome were published, sketching a highly intricate map of known and uncharted coding and regulatory elements (International Human Genome Sequencing Consortium et al., 2001; Venter et al., 2001). The identification and exhaustive description of the DNA sequence of a mammalian genome as published in 2001, though, is of limited use without a thorough knowledge of the function and regulation of the genetic elements in question (Orphanides and Reinberg, 2002). Genome biology is as much an exercise in reading (i.e. interpreting the genome and regulating its expression) as in writing (that is, changing the sequence and structure of the genome itself). The proof of the pudding, as is often suggested, is in the eating after all.

As tradition had it, gene expression obeyed a series of segregated and ordered steps (Orphanides and Reinberg, 2002). Cytoplasmic signaling to the nucleus, transcriptional regulation, RNA processing, mRNA trafficking and translation — all happened in a neat and linear sequence, one molecular process after another (Orphanides and Reinberg, 2002). The contemporary view of gene expression, however, is quite different, with several of these processes known to work together to correctly regulate the expression of target genes: the role of chromatin, not just in packing genetic information, but also in regulating its interpretation; the involvement of the transcriptional machinery in recruiting the

apparatus necessary for processing nascent RNAs; or the role played by pre-mRNA splicing in the promotion of transcriptional elongation (reviewed in Orphanides and Reinberg, 2002, Kornberg, 2007; Hager et al., 2009).

The three-dimensional organization of chromatin and its subsequent modulation in a transcriptional context is a major level of transcriptional regulation (Bickmore and van Steensel, 2013; Ferrai et al., 2010; de Wit and de Laat, 2012; Wendt and Grosveld, 2014). The formation and maintenance of DNA loops — formed over transcription initiation between enhancers and core promoters and stabilized by cohesin and the CCCTC-binding factor (CTCF) — seem to be key for regulating proper gene expression (Bickmore and van Steensel, 2013; Ferrai et al., 2010; de Wit and de Laat, 2012; Wendt and Grosveld, 2014; Lenhard et al, 2012).

From the moment a cell decides a certain gene is to be expressed, a complex cascade of molecular events must unfold. A series of recent studies has highlighted how astoundingly beautiful and intricate these processes are and to which degree the function and dynamic structure of the genome are integrated (Dowen et al., 2014; Chen et al., 2014; Liu et al., 2014; reviewed in Levine et al., 2014); this realization becomes easy to appreciate if one remembers the three-dimensional nature of the cellular nucleus and the necessary rules for unpacking, reading and expressing genetic information: in other words, the timings and rules that dictate how regulatory factors find their genomic binding partners. Once this molecular hide-and-seek has been played to fruition, the preinitiation complex (PIC) is formed by binding of sequence-specific regulatory proteins (i.e. transcription factors) and recruited coactivators, such as the Mediator complex, to specific DNA elements or transcription factor binding sites (TFBSs, such as enhancers), upstream of the transcription start site (TSS). This allows RNA Pol II to then bind to general transcription factors (GTFs) at the TSS (reviewed in Kornberg, 2007; Hager et al., 2009; Wendt and Grosveld, 2014; Levine et al., 2014). Recruited RNA Pol II will typically transcribe around 20-50 bp before pausing, a process controlled by the interacting factors DRB sensitivity inducing factor

(DSIF) and negative elongation factor (NELF) (reviwed in Adelman and Lis, 2012; Heidemann et al., 2013; Jonkers and Lis; 2015). The paused RNA Pol II molecules may then proceed towards productive elongation or terminate transcription (Adelman and Lis, 2012; Heidemann et al., 2013; Jonkers and Lis; 2015). Pause release and subsequent elongation is catalyzed by positive transcription elongation factor b (P-TEFb)-mediated phosphorylation of paused RNA Pol II (Adelman and Lis, 2012; Heidemann et al., 2013; Jonkers and Lis; 2015).

Constituting a 550 kDa complex of ten to twelve different subunits (depending on species), RNA Pol II catalyzes, in eukaryotes, the synthesis of mRNAs precursors, small nuclear RNAs (snRNAs) and microRNAs (reviwed in Hager et al., 2009; Kornberg, 2007; Wendt and Grosveld, 2014; Levine et al., 2014). The largest of these subunits, RPB1, contains a carboxy terminal domain (CTD) composed of approximately 52 repeats of a heptapeptidic consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPTSPS) (Allison et al., 1985; Corden et al., 1985). If individual aminoacids are numbered according to their position along the heptapeptide — $Y_1S_2P_3T_4S_5P_6S_7$ — we find three distinct serine residues: ser-2 (Ser2P), ser-5 (Ser5P) and ser-7 (Ser7P). The phosphorylation of each of these serines carries with it a regulatory role in transcriptional progression and is catalyzed by different kinases within different regulatory complexes (reviewed in Brookes and Pombo, 2009; Heidemann et al., 2013; Jonkers and Lis; 2015). Some histone-modifying enzymes use these dynamic CTD configurations to discriminate promoter-proximal from promoter-distal regions with, for example, a combination of lysine methylation marks signaling different gene regions (H3K4 trimethylation around the promoter region and H3K36 methylation across downstream transcribed regions) (Brookes and Pombo, 2009; Heidemann et al., 2013; Jonkers and Lis; 2015). Upon PIC formation, mediator promotes phosphorylation of RNA Pol II on Ser5P of RPB1 CTD by the transcription factor II D ($TF_{II}D$) (reviewed in Brookes and Pombo, 2009; Heidemann et al., 2013; Jonkers and Lis; 2015; Levine et al., 2014). This phosphorylation mark is maintained for part of the transcription cycle, but decreases as transcription progresses; the 5' mRNA capping

machinery uses CTD Ser5P residues to physically tether itself to a position close to the mRNA exit channel (Brookes and Pombo, 2009; Heidemann et al., 2013; Jonkers and Lis; 2015; Levine et al., 2014).

As RNA Pol II elongates, the balance between Ser5P and Ser2P changes (with a decrease of Ser5P and increase of Ser2P), as RNA Pol II is released from the paused state by the P-TEFb complex, which includes cyclin-dependent kinase 9 (CDK9), promoting the phosphorylation of RPB1 CTD Ser2 as well as that of NELF (which dissociates from RNA Pol II upon phosphorylation) (Heidemann et al., 2013; Levine et al., 2014; Jonkers and Lis; 2015). Similarly to Ser5P, Ser2P anchors RNA processing factors as well, interacting with polyadenylation machinery at mRNA 3' ends (Brookes and Pombo, 2009; Heidemann et al., 2013; Jonkers and Lis; 2015). Ser7 phosphorylation seems to be necessary for expression of snRNAs, but a fuller functional understanding of this phosphorylation mark is still in its infancy (reviewed in Heidemann et al., 2013).

Our understanding of RNA Pol II promoter-proximal pausing and its wider role in the regulation of gene expression is still incomplete. With the recent and current boom of functional genomic tools — from those integrating genome architecture and organization, to the visualization of *in vivo* single-molecule dynamics and their real-time three-dimensional movements — a more comprehensive bigger picture of genome wide expression regulation is emerging (Kieffer-Kwon et al., 2013; Liu et al., 2014; Chen et al., 2014; Crosetto et al., 2015; Schwartzman and Tanay, 2015). Most of the research into the mechanisms behind RNA Pol II pausing has focused on the "when" and "how" the necessary regulatory and effector machinery does its poised magic. However, some of the most intriguing and interesting insights into the overarching impact of this molecular phenomenon have come down to — and out of — the "where" it occurs. Our Waddingtonian brain is where we shall turn our attention to next.

2.4 RNA Polymerase II pausing in the brain

As has been a recurring theme throughout the second half of this introduction, we come back full circle to gene-environment interactions. Metazoans have evolved a complex myriad of mechanisms built specifically to translate developmental and environmental cues into appropriate transcriptional outputs (Wolf and Linden, 2012; Lyons and West, 2011; Puckett and Lubin, 2011). RNA Pol II pausing is such a mechanism (Gilmour and Lis, 1986). Transcription elongation was first identified as a rate-limiting step in gene expression in the 1970s and 1980s, but it was only with the analysis of the *Drosophila melanogaster* heat shock protein (Hsp) genes that already transcribing RNA Pol II was shown to accumulate downstream of gene promoters while associated with 20-50 bp-long nascent RNAs, naming this phenomenon, for the first time, as RNA Pol II "pausing" (Gilmour and Lis, 1986; Rougvie and Lis, 1988; Rougvie and Lis, 1990; Rasmussen and Lis, 1993). Throughout the years, other promoters were shown to display RNA Pol II pausing, in other species (including mammals) and ontogenetic contexts, from the transcriptional regulation of developmental genes to gene regulation in the adult organism (reviwed in Adelman and Lis, 2012; Levine et al., 2014; Jonkers and Lis; 2015). As a molecular mechanism that so beautiful and intuitively links activity-dependent transcriptional regulation to its upstream cues, RNA Pol II promoter-proximal stalling was naturally hypothesized as being at the basis of one or another biological phenomena. In the case of neuronal IEG expression, they just happened to be right.

In 2011, the lab of Serena Dudek published a paper in which it was shown, for the first time, that RNA Pol II pausing exists in the brain and that it is required for IEG rapid induction (further dividing IEGs into those with a rapid or delayed induction profile, that is, respectively, with or without promoter-proximal poised RNA Pol II), suggesting that this molecular mechanism may be involved in other cellular processes dependent on tightly regulated and fast transcriptional induction,

including the vast molecular and functional world of learning and memory (Saha et al., 2011). Aside from demonstrating that poised RNA Pol II does indeed play a role in neuronal activity-induced transcription as examined in *in vitro* neuronal cortical cultures (subjected to a prolonged treatment with tetrodotoxin [TTX], a sodium channel blocker, which upon washout induces quasi-synchronous neuronal activity), Saha et al. also detected RNA Pol II pausing in IEGs in the rat cortex and hippocampus *in vivo*, showing that this mechanism is additionally involved in RNA Pol II pausing-dependent IEG fast induction as a result of exposure to novel environments.

In chapter 3, we will explore the work of Saha el al. a bit further and frame, within the wider field of activity-induced gene transcription, our findings on the impact of learning on RNA Pol II pausing in the mouse striatum. We demonstrate that learning a motor skill shifts the phosphorylation dynamics of RNA Pol II, as well as its binding kinetics to IEGs, in the adult *in vivo* brain, providing a first instance of learning-dependent modulation of RNA Pol II pausing.

References

Adelman K and Lis JT (2012) **Promoter-proximal pausing of RNA Polymerase II: emerging roles in metazoans**. Nature Reviews Genetics 13: 720-731.

Alberini CM (2009) **Transcription factors in long-term memory and synaptic plasticity**. Physiological Reviews 89: 121-145.

Albin RL, Young AB and Penney JB (1989) **The functional anatomy of basal ganglia disorders**. Trends in Neuroscience 12: 366-375.

Alexander GE and Crutcher MD (1990) **Functional architecture of basal ganglia circuits: neural substrates of parallel processing**. Trends in Neuroscience 13: 266-271.

Allison LA, Moyle M, Shales M and Ingles CJ (1985) **Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA Polymerases**. Cell 42: 599-610.

Arendt D (2008) **The evolution of cell types in animals: emerging principles from molecular studies**. Nature Reviews Genetics 9: 868-882.

Balleine BW, Liljeholm M, Ostlund SB (2009). **The integrative function of the basal ganglia in instrumental conditioning**. Behavioural Brain Research 199: 43–52.

Barco A and Marie H (2011) **Genetic approaches to investigate the role of CREB in neuronal plasticity and memory**. Molecular Neurobiology 44: 330-349.

Bernard V, Normand E and Bloch B (1992) **Phenotypical characterization of the rat striatal neurons expressing muscarinic receptor genes**. Journal of Neuroscience 12: 3591-3600.

Bertran-Gonzalez J, Bosch C, Maroteaux M, Matamales M, Herve D, Valjent E and Girault JA (2008) **Opposing patterns of signaling activation in dopamine D1 and D2**

receptor-expressing striatal neurons in response to cocaine and haloperidol. Journal of Neuroscience 28: 5671-5685.

Bickmore WA and van Steensel B (2013) **Genome architecture: domain organization of interphase chromosomes**. Cell 152: 1270-1284.

Bolam JP, Hanley JJ, Booth PA and Bevan MD (2000) **Synaptic organization of the basal ganglia**. Journal of Anatomy 196: 527-542.

Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G and Silva AJ (1994) **Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein**. Cell 79: 59-68.

Boyden ES, Zhang F, Bamberg E, Nagel G and Deisseroth K (2005) **Millisecond-timescale, genetically targeted optical control of neural activity**. Nature Neuroscience 8: 1263-1268.

Brookes E and Pombo A (2009) **Modifications of RNA Polymerase II are pivotal in regulating gene expression states**. EMBO Reports 10: 1213-1219.

Buchen L (2010) **Neuroscience: Illuminating the brain**. Nature 465: 26-28.

Calabresi P, Picconi B, Tozzi A, Ghiglieri V and Di Filippo M (2014) **Direct and Indirect pathways of basal ganglia: a critical reappraisal**. Nature Neuroscience 17: 1022-1030.

Chahrour M, Jung SY, Shaw C, Zhou X, Wong, STC, Qin J and Zoghbi HY (2008) **MeCP2, a key contributor to neurological disease, activates and represses transcription**. Science 320: 1224-1229.

Chakalova L and Fraser P (2010) **Organization of Transcription**. Cold Spring Harbor Perspectives on Biology 2: 1-15.

Chen J, Zhang Z, Li L, Chen BC, Revyakin A, Hajj B, Legant W, Dahan M, Lionnet T, Betzig E, Tjian R and Liu Z (2014) **Single-molecule dynamics of enhanceosome assembly in embryonic stem cells**. Cell: 156: 1274-1285.

Corden JL, Cadena DL, Ahearn JM Jr and Dahmus ME (1985) **A unique structure at the carboxyl terminus of the largest subunit of eukaryotic RNA polymerase II**. Proceedings of the National Academy of Sciences USA 82: 7934-7938.

Crosetto N, Bienko M and van Oudenaarden A (2015) **Spatially resolved transcriptomics and beyond**. Nature Reviews Genetics 16: 57-66.

Crosio C, Heitz E, Allis CD, Borrelli E and Sassone-Corsi P (2003) **Chromatin remodeling and neuronal response: multiple signaling pathways induce specific histone H3 modifications and early gene expression in hippocampal neurons**. Journal of Cell Science 116: 4905-4914.

Cui G, Jun SB, Jin X, Pham MD, Vogel SS, Lovinger DM and Costa RM (2013) **Concurrent activation of striatal direct and indirect pathways during action initiation**. Nature 494: 238-242.

Dash PK, Hochner B and Kandel ER (1990) **Injection of the cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation**. Nature 345: 718-721.

de Wit Elzo and de Laat W (2012) **A decade of 3C technologies: insights into nuclear organization**. Genes & Development 26: 11-24.

DeLong MR (1990) **Primate models of movement disorders of basal ganglia origin**. Trends in Neuroscience 13: 281-285.

Deng JV, Rodriguiz RM, Hutchinson AN, Kim IH, Wetsel WC and West AE (2010) **MeCP2 in the nucleus accumbens contributes to neural and behavioral responses to psychostimulants**. Nature Neuroscience 13: 1128-1136.

Deneris ES and Hobert O (2014) **Maintenance of postmitotic neuronal cell identity**. Nature Neuroscience 17: 899-907.

Dowen JM, Fan ZP, Hnisz D, Ren G, Abraham BJ, Zhang LN, Weintraub AS, Schuijers J, Lee TI, Zhao K and Young RA (2014) **Control of cell identity genes occurs in insulated neighborhoods in mammalian chromosomes**. Cell: 159: 374-387.

Durieux PF, Bearzatto B, Guiducci S, Buch T, Waisman A, Zoli M, Schiffmann SN and de Kerchove d'Exaerde A (2009) **D2R striatopallidal neurons inhibit both locomotor and drug reward processes**. Nature Neuroscience 12: 393-395.

Durieux PF, Schiffmann SN and de Kerchove d'Exaerde A (2011) **Targeting neuronal populations of the striatum**. Frontiers in Neuroanatomy 5: 1-9.

Ena S, de Kerchove d'Exaerde A and Schiffmann SN (2011) **Unraveling the differential functions and regulation of striatal neuron sub-populations in motor control, reward, and motivational processes**. Frontiers in Behavioral Neuroscience 5: 1-10.

Faulk C and Dolinoy DC (2011) **Timing is everything: the when and how of environmentally induced changes in the epigenome of animals**. Epigenetics 6: 791-797.

Feng J, Shao N, Szulwach KE, Vialou V, Huynh J, Zhong C, Le T, Ferguson D, Cahill ME, Li Y, Koo JW, Ribeiro E, Labonte B, Laitman BM, Estey D, Stockman V, Kennedy P, Couroussé T, Mensah I, Turecki G, Faull KF, Ming GL, Song H, Fan G, Casaccia P, Shen L, Jin P and Nestler EJ. (2015) **Role of TET1 and 5-hydroxymethylcytosine in cocaine action**. Nature Neuroscience 18: 536-544.

Ferrai C, Castro IJ, Lavitas L, Chotalia M and Pombo A (2010) **Gene Positioning**. Cold Spring Harbor Perspectives in Biology 2:a000588.

Fischer A (2014) **Epigenetic memory: the Lamarckian brain**. The EMBO Journal 33: 945-967.

Fischer A, Sananbenesi, Wang X, Dobbin M and Tsai LH (2007) **Recovery of learning and memory is associated with chromatin remodeling**. Nature 447: 178-182.

Flavell SW and Greenberg ME (2008) **Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system**. Annual Review of Neuroscience 31: 563-590.

Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ Jr. and Sibley DR (1990) **D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons**. Science 250: 1429-1432.

Gerfen CR and Young WS III (1988) **Distribution of striatonigral and striatopallidal peptidergic neurons in both patch and matrix compartments: an in situ hybridization histochemistry and fluorescent retrograde tracing study**. Brain Research 460: 161-167.

Gilmour DS and Lis JT (1986) **RNA polymerase II interacts with the promoter region of the non-induced *hsp70* gene in *Drosophila melanogaster* cells**. Molecular and Cellular Biology 6: 3984-3989.

Goldie BJ and Cairns MJ (2012) **Post-translational trafficking and regulation of neuronal gene expression**. Molecular Neurobiology 45: 99-108.

Graybiel AM and Grafton ST (2015) **The striatum: where skills and habits meet**. Cold Spring Harbor Perspectives in Biology 7:a021691.

Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME and Heintz N (2003) **A gene expression atlas of the central nervous system based on bacterial artificial chromosomes**. Nature 425: 917-925.

Gremel CM and Costa RM (2013) **Orbitofrontal and striatal circuits dynamically encode the shift between goal-directed and habitual actions**. Nature Communications 4: 1-12.

Guy J, Cheval H, Selfridge J and Bird AP (2011) **The role of MeCP2 in the brain**. Annual Review of Cell and Developmental Biology 27: 631-652.

Hager GL, McNally JG and Misteli T (2009) **Transcription dynamics**. Molecular Cell 35: 741-753.

Hamby ME, Coskun V and Sun YE (2008) **Transcriptional regulation of neuronal differentiation: The epigenetic layer of complexity**. Biochimica et Biophysica Acta 1779: 432:437.

Heidemann M, Hintermain C, Voβ K and Eick D (2013) **Dynamic phosphorylation patterns of RNA Polymerase II CTD during transcription**. Biochimica aet Biophysica Acta 1829: 55-62.

Hilário MRF and Costa RM (2008) **High on habits**. Frontiers in Neuroscience 2: 208-217.

Hilário MRF, Holloway T, Jin X and Costa RM (2012) **Different dorsal striatum circuits mediate action discrimination and action generalization**. European Journal of Neuroscience 35: 1105-1114.

Hikida T, Kimura K, Wada N, Funabiki K and Nakanishi S (2010) **Distinct roles of synaptic transmission in direct and indirect pathways to reward and aversive behavior**. Neuron 66: 896-907.

Hollander JA, Im HI, Amelio AL, Kocerha J, Bali P, Lu Q, Willoughby D, Wahlestedt C, Conkright MD and Kenny PJ (2010) **Striatal microRNA controls cocaine intake through CREB signaling**. Nature 466: 197-202.

Im HI, Hollander JA, Bali P and Kenny PJ (2010) **MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212**. Nature Neuroscience 13: 1120-1127.

Ince E, Ciliax BJ and Levey AI (1997) **Differential expression of D1 and D2 dopamine and m4 muscarinic acetylcholine receptor proteins in identified striatonigral neurons**. Synapse 27: 357-366.

International Human Genome Sequencing Consortium et al. (2001) **Initial sequencing and analysis of the human genome**. Nature 409: 860-921.

Jin X, Tecuapetla F and Costa RM (2014) **Basal ganglia subcircuits distinctively encode the parsing and concatenation of action sequences**. Nature Neuroscience 17: 423-430.

Joel D and Weiner I (1994) **The organization of the basal ganglia-thalamo-cortical circuits: open interconnected rather than closed segregated**. Neuroscience 63: 363-379.

Johansen KM and Johansen J (2006) **Regulation of chromatin structure by histone H3S10 phosphorylation**. Chromosome Research 14: 393-404.

Jonkers I and Lis JT (2015) **Getting up to speed with transcription elongation by RNA polymerase II**. Nature Reviews Molecular Cell Biology 16: 167-177.

Kawaguchi Y, Wilson CJ, Augood SJ and Emson PC (1995) **Striatal interneurons: chemical, physiological, and morphological characterization**. Trends in Neuroscience 18: 527-535.

Kieffer-Kwon KR, Tang Z, Mathe E, Qian J, Sung MH, Li G, Resch W, Baek S, Pruett N, Grøntved L, Vian L, Nelson S, Zare H, Hakim O, Reyon D, Yamane A, Nakahashi H, Kovalchuk AL, Zou J, Joung JK, Sartorelli V, Wei CL, Ruan X, Hager GL, Ruan Y and Casellas R (2013) **Interactome maps of mouse gene regulatory domains reveal basic principles of transcriptional regulation**. Cell: 155: 1507-1520.

Kim TK, Hemberg M, Gray JM, Costa AM, Bear DM, Wu J, Harmin DA, Laptewicz M, Barbara-Haley K, Kuersten S, Markenscoff-Papadimitriou E, Kuhl D, Bito H, Worley PF, Kreiman G and Greenberg ME (2010) **Widespread transcription at neuronal activity-regulated enhancers**. Nature 465: 182-187.

Kornberg RD (2007) **The molecular basis of eukaryotic transcription**. Proceedings of the National Academy of Sciences USA 104: 12955-12961.

Krebs JE, Goldstein ES and Kilpatrick ST (2014) **Genes XI**. Jones and Bartlett Publishers.

Kreitzer AC and Malenka RC (2008) **Striatal plasticity and basal ganglia circuit function**. Neuron 60: 543-554

Kravitz AV, Freeze BS, Parker PRL, Kay K, Thwin MT, Deisseroth K and Kreitzer AC (2010) **Regulation of parkinsonian motor behaviours by optogenetic control of basal ganglia circuitry**. Nature 466: 622-626.

Kravitz AV, Tye LD and Kreitzer AC (2012) **Distinct roles for direct and indirect pathway striatal neurons in reinforcement**. Nature Neuroscience 15: 816-818

Kumar A, Choi K, Renthal W, Tsankova NM, Theobald DEH, Truong H, Russo SJ, LaPlant Q, Sasaki TS, Whistler KN, Neve RL, Self DW and Nestler EJ (2005) **Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum**. Neuron 48: 303-314.

Le Moine C, Normand E and Bloch B (1991) **Phenotypical characterization of the rat striatal neurons expressing the D1 dopamine receptor gene**. Proceedings of the National Academy of Sciences USA 88: 4205-4209.

Lenhard B, Sandelin A and Carninci P (2012) **Metazoan promoters: emerging characteristics and insights into transcriptional regulation**. Nature Reviews Genetics 13: 233-245.

Levine AA, Guan Z, Barco A, Xu S, Kandel ER and Schwartz JH (2005) **CREB-binding protein controls response to cocaine by acetylating histones at the fosB promoter in the mouse striatum**. Proceedings of the National Academy of Sciences USA 102: 19186-19191.

Levine M, Cattoglio C and Tjian R (2014) **Looping back to leap forward: transcription enters a new era**. Cell 157: 13-25.

Liu Z, Legant WR, Chen B, Li L, Grimm JB, Davis LD, Betzig E and Tjian R (2014) **3D imaging of Sox2 enhancer clusters in embryonic stem cells**. eLife 3:e04236.

Lobo MK, Karsten SL, Gray M, Geschwind DH and Yang XW (2006) **FACS-array profiling of striatal projection neuron subtypes in juvenile and adult mouse brains**. Nature Neuroscience 9: 443:452.

Lobo MK (2009) **Molecular profiling of striatonigral and striatopallidal medium spiny neurons: past, present and future**. International Review of Neurobiology 89: 1-35.

Lyons MR and West AE (2011) **Mechanisms of specificity in neuronal activity-regulated gene transcription**. Progress in Neurobiology 94: 259-295.

Macpherson T, Morita M and Hikida T (2014) **Striatal direct and indirect pathways control decision-making behavior**. Frontiers in Psychology 5: 1-7.

Malik AN, Vierbuchen T, Hemberg M, Rubin AA, Ling E, Couch CH, Stroud H, Spiegel I, Farh KK, Harmin DA and Greenberg ME (2014) **Genome-wide identification and characterization of functional neuronal activity-dependent enhancers**. Nature Neuroscience 17: 1330-1339.

Matamales M and Girault JA (2011) **Signaling from the cytoplasm to the nucleus in striatal medium-sized spiny neurons**. Frontiers in Neuroanatomy 5: 1-13.

Meaney MJ and Ferguson-Smith AC (2010) **Epigenetic regulation of the neural transcriptomes: the meaning of the marks**. Nature Neuroscience 13: 1313-1318.

Mellén M, Ayata P, Dewell S, Kriaucionis S and Heintz N (2012) **MeCP2 binds to 5hmC enriched within active genes and accessible chromatin in the nervous system**. Cell 151: 1417-1430.

Mink JW (1996) **The basal ganglia: focused selection and inhibition of competing motor programs**. Progress in Neurobiology 50: 381-425.

Montminy MR, Sevarino KA, Wagner JA, Mandel G, Goodman RH (1986) **Identification of a cyclic-AMP-responsive element within the rat somatostatin gene**. Proceedings of the National Academy of Sciences USA 83: 6682-6686.

Nan X, Meehan RR and Bird AP (1993) **Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2**. Nucleic Acids Research 21: 4886-4892.

Orphanides G and Reinberg D (2002) **A unified theory of gene expression**. Cell 108: 439-451.

Parent A (1990) **Extrinsic connections of the basal ganglia**. Trends in Neuroscience 13: 254-258.

Puckett RE and Lubin FD (2011) **Epigenetic mechanisms in experience-driven memory formation and behavior**. Epigenomics 3: 649-664.

Qureshi IA and Mehler MF (2014) **An evolving view of epigenetic complexity in the brain**. Philosophical Transactions of the Royal Society B 369: 1-7.

Rasmussen EB and Lis JT (1993) **In vivo transcriptional pausing and cap formation on three *Drosophila* heat shock genes**. Proceedings of the National Academy of Sciences USA 90: 7923-7927.

Riccio A (2010) **Dynamic epigenetic regulation in neurons: enzymes, stimuli and signaling pathways**. Nature Neuroscience 13: 1330-1337.

Rougvie AE and Lis JT (1988) **The RNA Polymerase II molecule at the 5' end of the uninduced *hsp70* gene of *D. melanogaster* is transcriptionally engaged**. Cell 54: 795-804.

Rougvie AE and Lis JT (1990) **Postinitiation transcriptional control in *Drosophila melanogaster***. Molecular and Cellular Biology 10: 6041-6045.

Rudenko A, Dawlaty MM, Seo J, Cheng AW, Meng J, Le T, Faull KF, Jaenisch R and Tsai LH (2013) **TET1 is critical for neuronal activity-regulated gene expression and memory extinction**. Neuron 79: 1109-1122.

Saha RN, Wissink EM, Bailey ER, Zhao M, Fargo DC, Hwang JY, Daigle KR, Fenn JD, Adelman K and Dudek SM (2011) **Rapid activity-induced transcription of *Arc* and other IEGs relies on poised RNA Polymerase II**. Nature Neuroscience 14: 849-856.

Saha RN and Dudek SM (2013) **Splitting hares and tortoises: a classification of neuronal immediate early gene transcription based on poised RNA Polymerase II**. Neuroscience 247: 175-181.

Schiffmann SN, Jacobs O and Vanderhaeghen JJ (1991) **Striatal restricted adenosine A2 receptor (RDC8) is expressed by encephalin but not by substance P neurons: an in situ hybridization histochemistry study**. Journal of Neurochemistry 57: 1062-1067.

Schübeler D (2015) **Function and information content of DNA methylation**. Nature 517: 321-326.

Schwartzman O and Tanay A (2015) **Single-cell epigenomics: techniques and emerging applications**. Nature Reviews Genetics 16: 716-726.

Shuen JA, Chen M, Gloss B and Calakos N (2008) **Drd1a-tdTomato BAC transgenic mice for simultaneous visualization of medium spiny neurons in the direct and indirect pathways of the basal ganglia**. Journal of Neuroscience 28: 2681-2685.

Skene PJ, Illingworth RS, Webb S, Kerr ARW, James KD, Turner DJ, Andrews R and Bird AP (2010) **Neuronal MeCP2 is expressed at near histone-octamer levels and globally alters the chromatin state**. Molecular Cell 37: 457-468.

Steiner H and Tseng KY (2010) **Handbook of basal ganglia structure and function**. Academic Press - Elsevier.

Sturtevant AH (1913) **The linear arrangement of six sex-linked factors in Drosophila, as shown by their mode of association**. Journal of Experimental Zoology 14: 43-39.

Trapnell C (2015) **Defining cell types and states with single-cell genomics**. Genome Research 25: 1491-1498.

Venter J et al. (2001) **The sequence of the human genome**. Science 291: 1304-1351.

Waddington CH (1942) **Canalization of development and the inheritance of acquired characters**. Nature 150: 563-565.

Watson JD and Crick FHC (1953) **Molecular structure of nucleic acids**. Nature 171: 737-738.

Wendt KS and Grosveld FG (2014) **Transcription in the context of the 3D nucleus**. Current Opinion in Genetics & Development 25: 62-67.

West AE and Greenberg ME (2011) **Neuronal activity-regulated gene transcription in synapse development and cognitive function**. Cold Spring Harbor Perspectives in Biology 3: 1-21.

Wolf C and Linden DEJ (2012) **Biological pathways to adaptability — interactions between genome, epigenome, nervous system and environment for adaptive behavior**. Genes, Brain & Behavior 11: 3-28.

Yin HH and Knowlton BJ (2006) **The role of the basal ganglia in habit formation**. Nature Reviews Neuroscience 7: 464–476.

Yin HH, Mulcare SP, Hilário MRF, Clouse E, Holloway T, Davis MI, Hansson AC, Lovinger DM and Costa RM (2009) **Dynamic reorganization of striatal circuits during the acquisition and consolidation of a skill**. Nature Neuroscience 12: 333-341.

Yizhar O, Fenno LE, Davidson TJ, Mogri M and Deisseroth K (2011) **Optogenetics in neural systems**. Neuron 71: 9-34.

Yu C, Gupta J, Chen J and Yin HH (2009) **Genetic deletion of A_{2A} adenosine receptors in the striatum selectively impairs habit formation**. Journal of Neuroscience 29: 15100-15103.

Zaratiegui M, Irvine DG and Martienssen RA (2007) **Noncoding RNAs and gene silencing**. Cell 128: 763-776.

"Dance, dance, otherwise we are lost."

Pina Bausch

Chapter II

Differential role of striatonigral and striatopallidal dorsolateral striatum neurons in positive reinforcement

Manuscript under revision: Vicente AM*, Galvão-Ferreira P*, Tecuapetla F and Costa RM. **Direct and indirect dorsolateral striatum pathways reinforce different action strategies**. *These authors contributed equally to this work.

Author contributions: Ana M Vicente, Pedro Galvão-Ferreira and Rui M Costa designed the experiments. Ana M Vicente, Pedro Galvão-Ferreira and Fatuel Tecuapetla performed the experiments. Ana M Vicente and Pedro Galvão-Ferreira analyzed the data.

Abstract

It has been proposed that striatal output pathways have opposing roles in action reinforcement, with direct striatonigral neurons supporting positive reinforcement, and indirect striatopallidal neurons coding for action avoidance. Using an optogenetics instrumental task, we uncovered that self-stimulation of either pathway in dorsolateral striatum leads to positive reinforcement, but supports different action strategies. Activation of striatonigral neurons produced rapid action-specific reinforcement, while striatopallidal neuron self-stimulation resulted in generalization to similar actions, and less sensitivity to action-stimulation contingency. These results contribute to a new model of basal ganglia function, in which the striatonigral and striatopallidal output pathways are not cleanly dichotomic, but work concomitantly to regulate action performance.

Taking advantage of the genetic identifiability of striatonigral and striatopallidal neurons provided by this system of dopamine D1 and dopamine D2 receptor-directed expression of Channelrhodopsin-2 (ChR2) and yellow fluorescent protein (YFP), we attempted to join circuit-specific activity manipulation and the analysis of its activity-dependent transcriptional plasticity. Fluorescence-activated cell sorting (FACS) was performed on striatal tissue removed from optogenetically-controlled D1- and D2-YFP/ChR2-YFP mice and total RNA extracted from these cell populations. We were unable to extract high quality RNA from FACS-isolated cells, but suggest possible future avenues for neuronal isolation of genetically identified circuits.

Introduction

The basal ganglia, and the dorsal striatum in particular, are critical for action reinforcement (Albin et al., 1989; DeLong, 1990; Mink, 1996; Yin and Knowlton, 2006). The dorsal striatum, which can be further subdivided into dorsomedial (DMS) and dorsolateral (DLS) striatum, is mainly composed of two subpopulations of morphologically identical striatal medium spiny projection neurons (MSNs): dopamine D_1 receptor-expressing MSNs that reach directly the basal ganglia output nuclei and constitute the striatonigral or direct pathway (dMSNs); and dopamine D_2 receptor-expressing MSNs that constitute the striatopallidal or indirect pathway (iMSNs) (Gerfen et al., 1990). It has been suggested that striatonigral and striatopallidal neurons have opposing roles in reinforcement, with striatonigral neurons being important to learn positive reinforcement and indirect pathway neurons to learn to avoid undesired actions (Go/No-Go) (Frank et al., 2004). Consistently, it has been shown that optogenetic self-stimulation of striatonigral neurons in DMS leads to reinforcement of actions that lead to stimulation, while self-stimulation of striatopallidal neurons leads to avoidance of actions that lead to stimulation (Kravitz et al., 2012). However, in DLS, which has been implicated in the consolidation of well-trained actions and in habit formation (Yin and Knowlton, 2006; Yin et al., 2009), both projection pathways are active during lever pressing for reward (Cui et al., 2013; Jin et al., 2014). Furthermore, extensive skill training leads to long-lasting potentiation of glutamatergic inputs into both d- and iMSNs (Yin et al., 2009). It has also been shown that striatal-specific deletion of $A_{2A}R$, which abolishes long-term potentiation onto iMSNs, impairs habit formation (Yu et al., 2009). This functional characterization of the direct and indirect pathways is taking the basal ganglia field into a renewed understanding of the mechanisms of action of dMSNs and iMSNs. To build on this, our data suggest that in DLS both d- and iMSNs are involved in action learning and positive reinforcement, but support different action strategies.

Results

2.1 Role of DLS striatonigral and striatopallidal neurons in action reinforcement

To investigate the role of DLS striatonigral and striatopallidal neurons in action reinforcement, we used a self-stimulation paradigm where we activated specifically each pathway upon lever pressing.

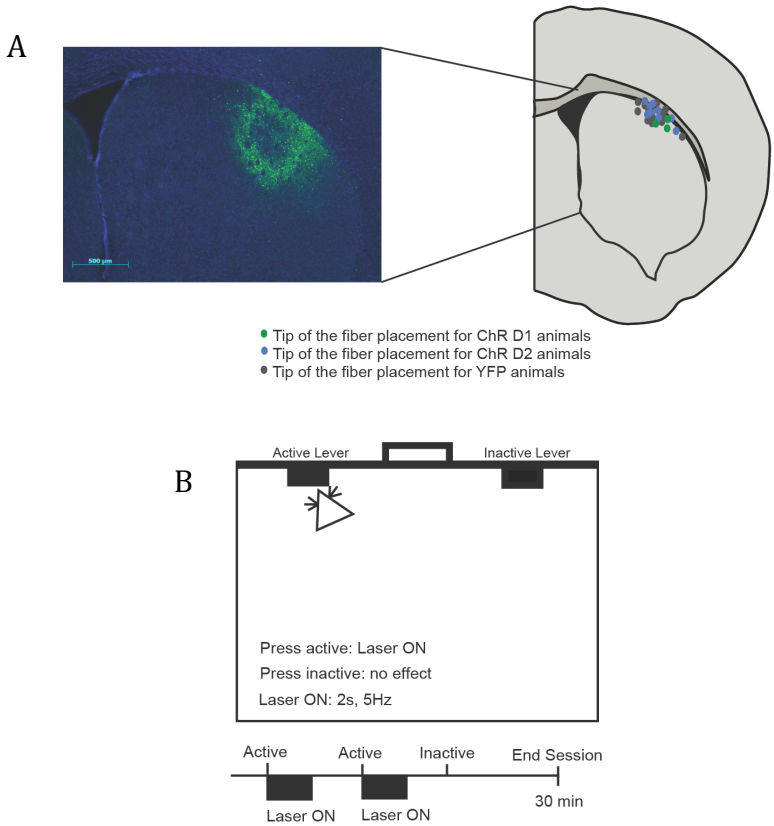


Figure 2.1: **Optogenetic self-stimulation of striatonigral and striatopallidal DLS neurons**. A) Schematics and representative histology slice of injection and fiber placement sites in DLS. B) Schematics of the operant box and the behavioral paradigm, including light stimulation protocol.

We injected a virus expressing Channelrhodopsin-2 (ChR, AAV2/1) in a Cre-dependent manner into DLS of mice expressing Cre recombinase in either striatonigral (D_1-Cre, line EY217; Gong et al., 2007) or striatopallidal neurons (D_2-Cre, line ER43; Gong et al., 2007) (figure 2.1A). Two weeks after infection, animals were trained in an operant box with two levers (figure 2.1B): an active lever where pressing resulted in the delivery of blue light (473 nm) into DLS, and an inactive lever (no light delivered).

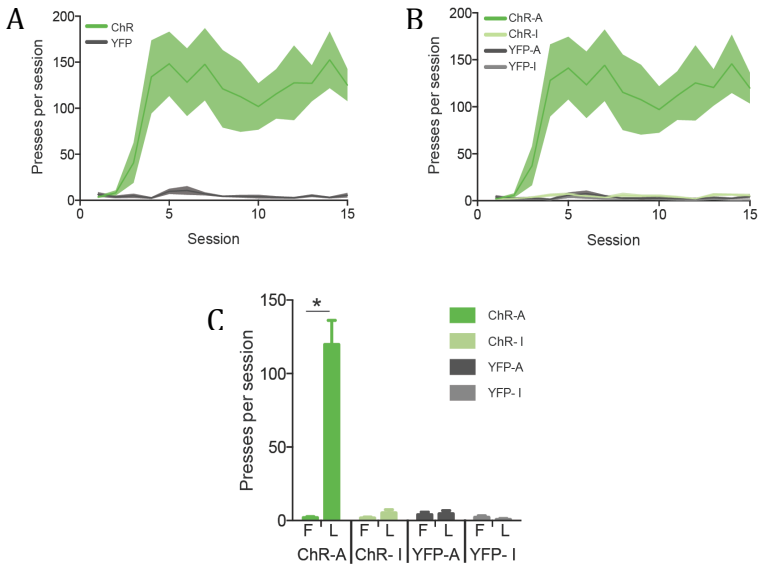


Figure 2.2: **Acquisition of lever pressing for ChR D_1-Cre animals infected animals and YFP controls.** A) Total number of lever presses per day for each group: ChR D_1-Cre (green line, n=6) and YFP controls (grey line, n=6) (Main effect of D_1 training $F_{14,140}$=4.987, P<0.0001; ChR effect $F_{1,10}$=20.67, P=0.0011; Interaction $F_{14,140}$=4.883, P<0.0001.). B) Lever presses in the active versus the inactive lever of each group (Figure 1d - Main effect of D_1 training $F_{42,280}$=5.143, P<0.0001; lever and ChR effect $F_{3,20}$=21.21, P<0.0001; Interaction $F_{42,280}$=4.760, P<0.0001). C) Difference in pressing from the first to the last day of training for ChR and YFP D_1-Cre animals, for active and inactive levers (Main effect of D_1 training $F_{1,20}$=53.18, P<0.0001; lever and ChR effect $F_{3,20}$=45.38, P<0.0001; Interaction $F_{3,20}$=50.14, P<0.0001. Post hoc ChR active first day versus ChR active last day: P<0.0001). Mean ± s.e.m plotted in all graphs; A: active lever; I: inactive lever; F: first day of training; L: last day of training.

Self-paced reinforced lever presses resulted in the delivery of 10 pulses of light (for 2 sec, at 5Hz, 10ms wide pulses). This stimulation frequency was chosen because it is similar to the endogenous activity of MSNs (Jin et al., 2014; Tecuapetla et al., 2014). Each session lasted 30 minutes with no maximum number of reinforcers. Both groups of ChR-expressing mice increased the number of presses with training, and pressed significantly more than YFP controls (figure 2.2A, $F_{1,10}$=20.67, P=0.0011; figure 2.3A, $F_{1,17}$=5.845, P=0.0271). Consistent with previous studies, D_1-Cre animals acquired lever pressing rather rapidly, and pressed the active lever significantly more than the inactive lever (figure 2.2B, $F_{3,20}$=21.21, P<0.0001; figure 2.2C, First versus last day of active lever with ChR: P<0.0001).

On the other hand, D_2-Cre animals expressing ChR were slower in acquisition, and showed a significant increase in lever pressing for both the active and the inactive levers (figure 2.3B, $F_{3,34}$=3.111, P=0.0390; figure 2.3C, First versus last day for both active and inactive lever with ChR: P<0.05).

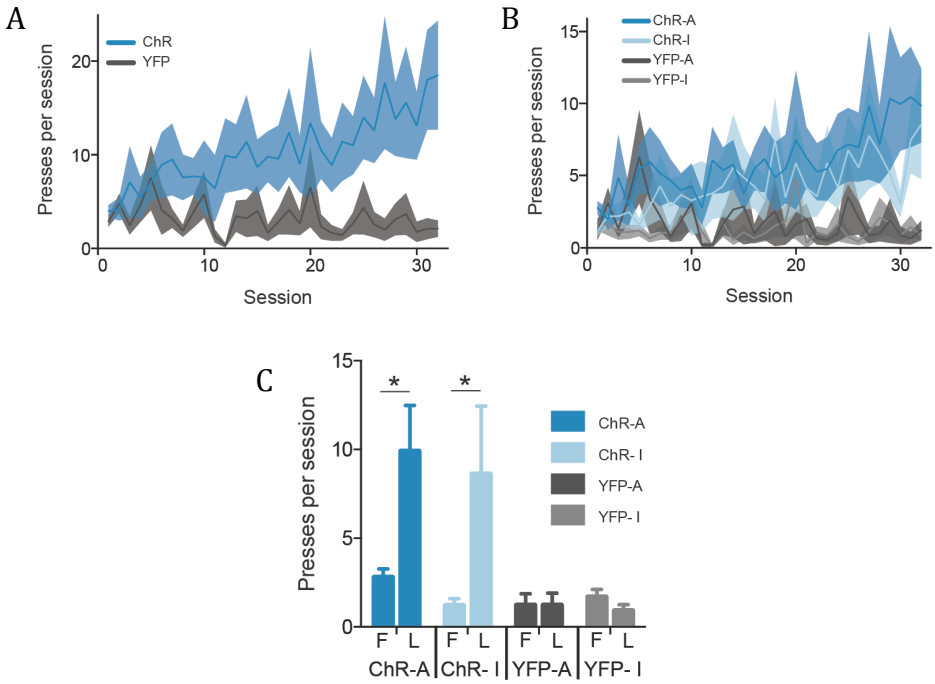


Figure 2.3: **Acquisition of lever pressing for ChR D_2-Cre animals and YFP controls.** A) Total number of presses per day for each group: ChR D_2-Cre animals (n=10) and YFP controls (n=9) (Main effect of D_2 training $F_{31,527}$=1.120, P=0.3026; ChR effect $F_{1,17}$=5.845, P=0.0271; Interaction $F_{31,527}$=1.505, P=0.0411). B) Lever pressing in both active and inactive levers (Main effect of D_2 training $F_{31,1054}$=1.516, P=0.0355; lever and ChR effect $F_{3,34}$=3.111, P=0.0390; Interaction $F_{93,1054}$=1.093, P=0.2643). C) Difference in pressing from the first to the last day of training for ChR and YFP D_2-Cre animals, for active and inactive levers (Main effect of D_2 training $F_{1,34}$=8.282, P=0.0069; lever and ChR effect $F_{3,34}$=3.858, P=0.0177; Interaction $F_{3,34}$=3.442, P=0.0274. Post hoc ChR active first day versus ChR active last day: P<0.05; ChR inactive first day versus ChR inactive last day: P<0.05). Mean ± s.e.m plotted in all graphs; A: active lever; I: inactive lever; F: first day of training; L: last day of training.

2.2 Reinforcement of different action strategies by striatonigral and striatopallidal DLS neurons

These data suggest that stimulation of both d- and iMSNs in DLS is reinforcing and not aversive, but leads to the development of different action strategies. To better characterize this dichotomy, we calculated the probability of pressing the active versus the inactive lever in every session. D_1-Cre animals expressing ChR showed a steady increase in the probability of pressing the active lever with training versus the probability of pressing the inactive lever ($F_{1,10}$=688.3, P<0.0001, figure 2.4A). On the other hand, D_2-Cre animals also had a higher probability of pressing the active than the inactive lever ($F_{1,18}$=6.961, P=0.0167, figure 2.5A) but this was mainly due to differences early in training (interaction $F_{LeverxTraining\ time}$ $F_{31,558}$=1.903), and eventually converged to a similar probability of pressing either lever (Posthocs not different for last days).

To further investigate if this equal pressing of both levers resulted from generalization of lever pressing, or from avoidance of the active lever and shifting to the inactive lever after an active press, we calculated the probability of transition from an active stimulated lever press to a subsequent active press (or conversely, to an inactive press, figure 2.4B and figure 2.5B). By the end of training, D_1-Cre animals reached a very high probability of pressing the active lever again after a previous reinforced active press ($F_{1,10}$=310.9, P<0.0001, figure 2.4B).

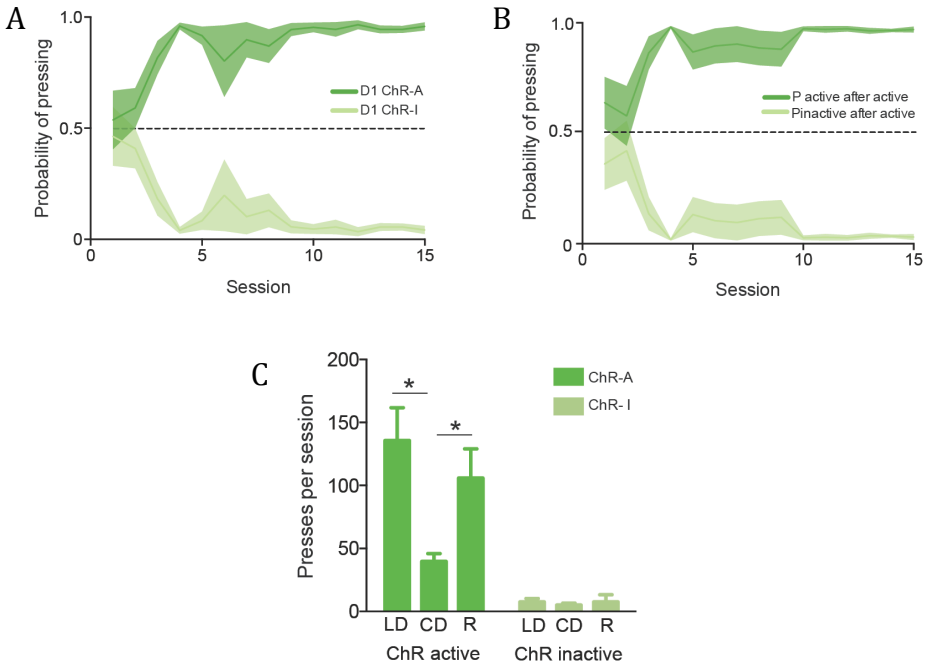


Figure 2.4: **Self-stimulation of striatonigral DLS neurons supports the development of action-specific reinforcement.** A) Probability of pressing the active versus the inactive lever for D_1-Cre animals (Main effect of D_1 training $F_{14,140}=3.447x10^{-14}$, $P>0.9999$; lever effect $F_{1,10}=688.3$, $P<0.0001$; Interaction $F_{14,140}=7.367$, $P<0.0001$. Post hoc p(active) versus p(inactive): $P<0.0001$ sessions 3-15). B) Probability of transition from an active lever press to a subsequent active lever press (versus an inactive press) for ChR D_1-Cre animals (Main effect of D_1 training $F_{14,140}=1.752x10^{-14}$, $P>0.9999$; lever effect $F_{1,10}=310.9$, $P<0.0001$; Interaction $F_{14,140}=7.485$, $P<0.0001$. Post hoc p(active after active) versus p(inactive after active): $P<0.0001$ sessions 3-15). C) Contingency degradation and reinstatement for D_1-Cre animals (Main effect of D_1 contingency degradation $F_{2,20}=6.410$, $P=0.0071$; lever effect $F_{1,10}=45.68$, $P=0<0001$; Interaction $F_{2,20}=5.687$, $P=0.0111$. Post hoc ChR active Last day versus ChR active CD: $P<0.001$; ChR active CD versus ChR active R2: $P<0.01$). Mean ± s.e.m plotted in all graphs; A: active lever; I: inactive lever; LD: last day of training; CD: contingency degradation day; R: reinstatement day.

D_2-Cre animals presented a slight but significantly higher probability of pressing the active lever after a reinforced active press throughout training

($F_{1,18}$=13.38, P=0.0018, although close to chance, figure 2.5A), indicating that D_2-Cre mice were not just shifting to the inactive lever after an active lever press and then shifting back.

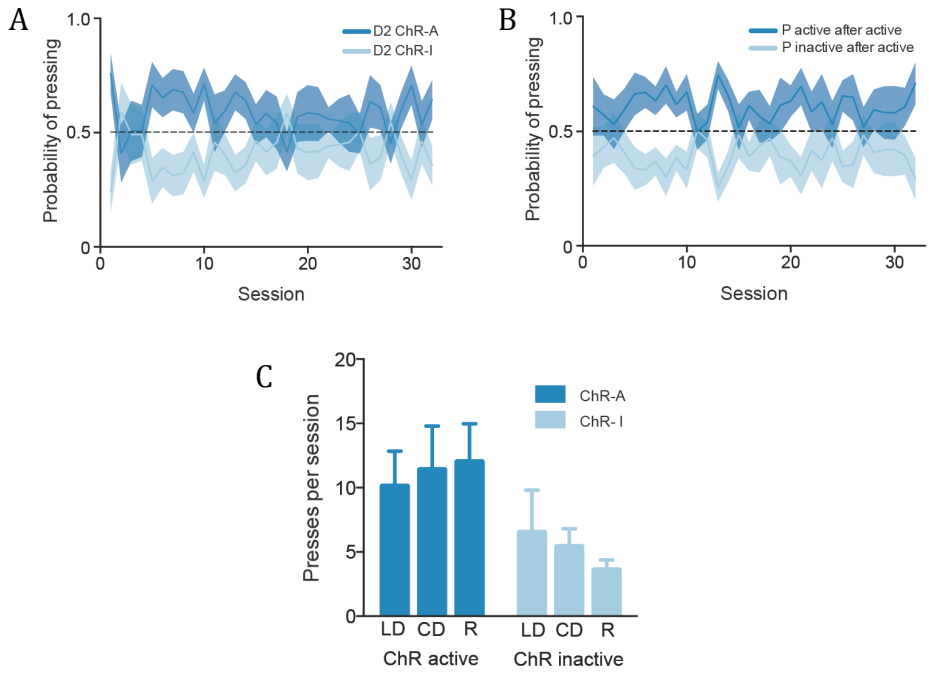


Figure 2.5: **Self-stimulation of striatopallidal DLS neurons supports the development of a stimulus-response habit that generalizes to similar actions.** A) Probability of pressing active lever versus the inactive lever for D_2-Cre animals (Main effect of D_2 training $F_{31,558}=5.904x10^{-15}$, P>0.9999; lever effect $F_{1,18}=6.961$, P=0.0167; Interaction $F_{31,558}=1.903$, P=0.0026. Post hoc p(active) versus p(inactive): P<0.01 for session 1). B) Probability of transition from an active lever press to a subsequent active lever press (versus a following inactive press) for ChR D_2-Cre animals (Main effect of D_2 training $F_{31,558}=5.696x10^{-15}$, P>0.9999; lever effect $F_{1,18}=13.38$, P=0.0018; Interaction $F_{31,558}=1.176$, P=0.2362. Post hoc p(active) versus p(inactive): P<0.01 for session 13; P<0.05 for session 32). C) Contingency degradation and reinstatement for D_2-Cre animals (Main effect of D_2 contingency degradation $F_{2,36}=0.09552$, P=0.9091; lever effect $F_{1,18}=3.295$, P=0.0862; Interaction $F_{2,36}=1.331$, P=0.2769). Mean ± s.e.m plotted in all graphs; A: active lever; I: inactive lever; LD: last day of training; CD: contingency degradation day; R: reinstatement day.

The data above suggest that self-stimulation of iMSNs leads to generalization between both levers, which is consistent with a role of these neurons in habit formation rather than goal-directed actions (Hilário et al., 2007; Hilário et al., 2012). To evaluate if the actions of both groups were goal-directed and therefore sensitive to changes in the contingency between action and outcome, or habitual and therefore less sensitive to changes in contingency, we performed a contingency degradation (CD) experiment. During contingency degradation, the light stimulation was delivered non-contingently upon lever pressing, with the same probability of reinforcement per unit of time as during training. Following CD, animals underwent contingency reinstatement, where pressing the active lever would again lead to the delivery of stimulation. D_1-Cre animals decreased the number of presses during the CD session (figure 2.4C, Last day versus CD for ChR-A animals: $P<0.001$), and resumed their lever pressing behavior during reinstatement (CD versus reinstatement for ChR-A animals: $P<0.01$). D_2-Cre animals, on the other hand, presented no changes in the number of presses during CD (figure 2.5C), suggesting that the lever pressing in these animals was habitual. The insensitivity to contingency degradation in iMSN-stimulated animals is unlikely to be due to a floor effect, since it has been previously shown that animals that press less tend to be more sensitive to contingency manipulation (Hilário et al., 2007).

Finally, to confirm that the differential lever pressing acquisition patterns observed in D1- and D2-cre animals did not stem from the different number of pairings between action and reinforcer, we analyzed the matched number of reinforcers between groups (figure 2.6A-B). As shown in figure 2.6A, after 250 reinforcers, D2-cre animals pressed both levers, while D1-cre animals pressed selectively the active lever; the same result may be observed if the cumulative number of presses for the first 25 and 250 reinforcers are individually quantified for D1- (figure 2.6B, left panel) and D2-cre animals (figure 2.6B, right panel).

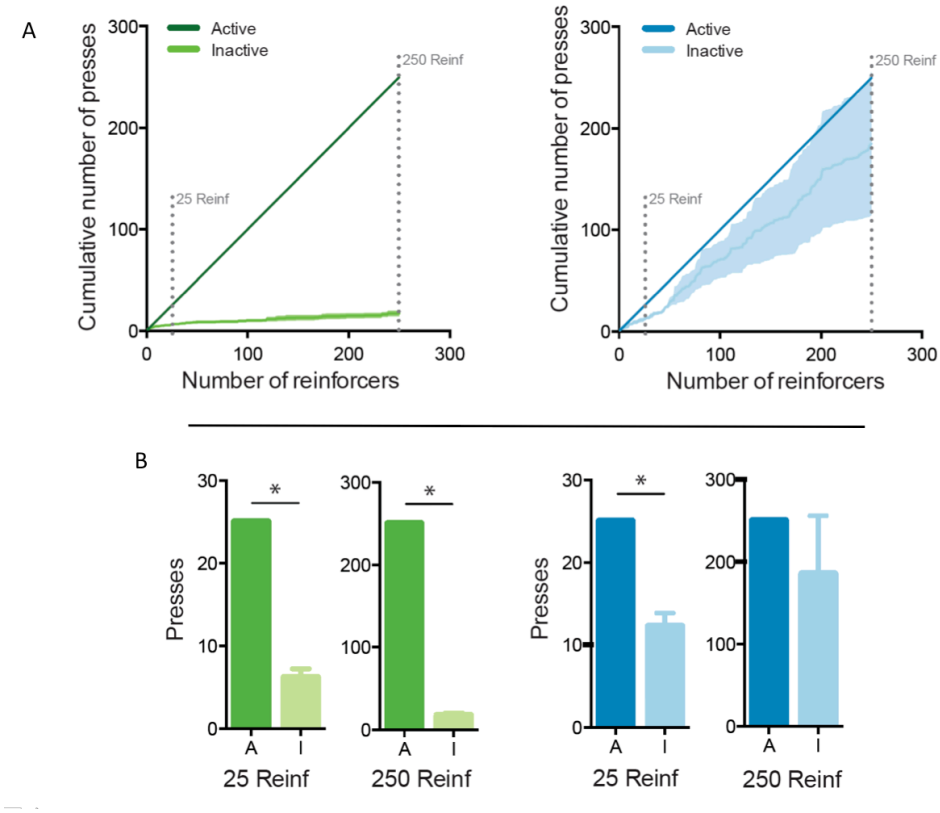


Figure 2.6: **Matched number of reinforcers for D1- and D2-cre animals**. A) Cumulative active and inactive presses for D1-cre (green) and D2-cre (blue) animals for the first 250 reinforcers earned. B) Cumulative number of presses for 25 and 250 reinforcers for D1-cre (green) and D2-cre (blue) animals.* denotes $p<0.005$

2.3 Fluorescence-activated cell sorting of optogenetically-controlled striatonigral and striatopallidal neurons

As mentioned in chapter I, the recent development of bacterial artificial chromosome (BAC)-carrying transgenic mice expressing fluorescent protein genes selectively in specific neuronal subtypes under cell type-specific promoters, has allowed the basal ganglia field to bring systems neuroscience and the study of transcriptional dynamics together under the experimental umbrella of circuit specificity (Lobo et al., 2006; reviewed in Okaty et al., 2011). Along with circuit activity manipulations as performed and described in the current chapter, transcriptional analyses of those same circuits became commonplace. Recently, fluorescence-activated cell sorting (FACS) and microarray analysis were applied to the striata of *Chrm4-EGFP*, *Drd1a-EGFP* and *Drd2-EGFP* mice to identify genes differentially expressed between striatonigral and striatopallidal neurons (Lobo et al., 2006). The creation of mice expressing Cre recombinase under the control of the Drd1a and Drd2 receptors (D1-Cre and D2-Cre mice; Gong et al., 2007) allowed for an equally precise *in vivo* probing of the neurophysiological functioning of each pathway, but it also made their cellular isolation for biochemical analysis possible.

By utilizing a similar surgical delivery of AAV2/1 viral vectors co-expressing ChR and YFP, but utilizing a different optogenetic approach (mice were placed in a 40cm x 40cm open field arena kept in a sound attenuating box for 20 minutes in each of 5 experimental days, and subject to 1mW blue laser stimulation at 14Hz for 10s, followed by 50s without laser stimulation, repeated 20 times to a total trial duration of 20 minutes, with one session per day), we attempted the identification of striatonigral and striatopallidal neurons via FACS (representative examples of ChR D_1-Cre and D_2-Cre sorted striata in figures 2.7B and 2.7C, respectively) to characterize the changes in transcriptional profile in these neurons induced by repeated optogenetic stimulation.

FACS is a specific type of flow cytometry technology that not only permits the selection of cellular populations based on several properties (such as size, granularity or the presence of a fluorescent signal), but also physically separates the selected cells, allowing for the extraction of near pure populations for the cellular properties selected by different FACS "gates" (i.e. a set of value limits for a certain properties that characterizes the population being sought) (BD Biosciences, 2000). Cellular populations are typically presented, in a first instance, as a function of cell size (as indicated by the forward-scattered light, FSC) and granularity (or internal cellular complexity, as indicated by the side-scattered light, SSC), with cellular debris easily visible as a spread of small size and high complexity particles (low FSC, high SSC; left panels of figure 2.7).

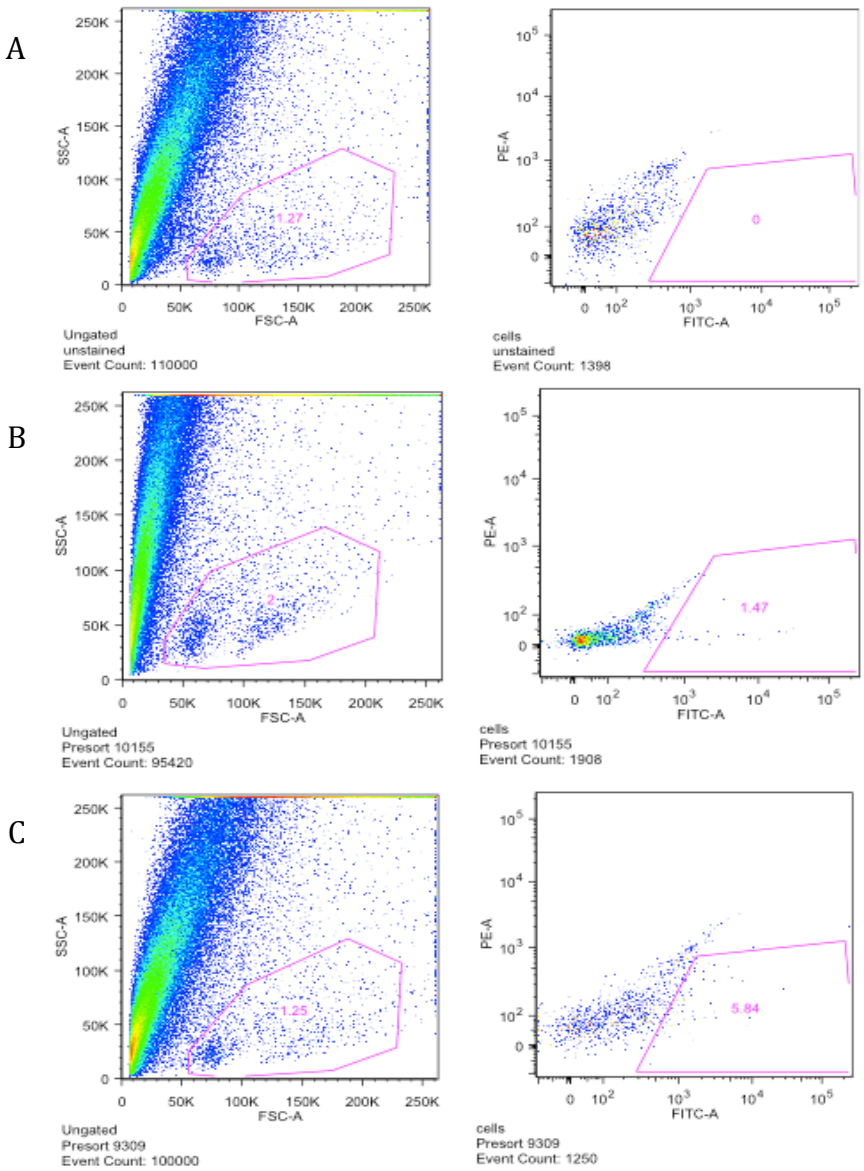


Figure 2.7: **FACS extraction of YFP$^+$ cells**. A) WT uninfected control sample. B) ChR D_1-Cre mouse. C) ChR D_2-Cre mouse. SSC: side-scattered light; FSC: forward-scattered light; FITC: fluorescein isothiocyanate, in this case functions as a proxy for YFP signal.

After optogenetic manipulation, total striata were dissected, dissociated with Papain enzyme, and subject to FACS. Neurons were gated in an FSC/SSC

plot according to the suspected size of the MSN population in question, with the percentage corresponding to gated/selected cells indicated (between 1-2% of total analyzed cells; left panels of figure 2.7A-C: 1.27%, 2% and 1.25%, respectively). As previously described (reviwed in Durieux et al., 2011; Ena et al., 2011), we confirm that morphologically, as visualized by FACS, striatonigral and striatopallidal neurons are virtually indistinguishable. Cells selected by the initial gate based on FSC/SSC properties were then gated according to the presence, or lack thereof, of fluorescence (as indicated by the fluorescein isothiocyanate [FITC] signal, in this case as a proxy for YFP signal, when compared to the phycoerythrin [PE] signal, indicating autofluorescence). In figure 2.7A, and as expected, no YFP^+ neurons are detected within the striata of an uninfected WT (i.e. Cre^-) mouse. In figures 2.7B and 2.7C, however, YFP^+ cells are detected (1.47% and 5.84% of FSC/SSC gated cells, respectively). Cells with a high FITC/YFP signal, but low PE/autofluorescence signal, were sorted, resulting in approximately 1000-2000 YFP^+ neurons per experimental animal. (To confirm YFP signal specificity, YFP^+ cells were re-sorted. The totality of the re-sorted population fell on the initial FSC/SSC gate [the same gate illustrated on the left panels of figures 2.7A-C], and within the FITC/PE gate [right panels of figures 2.7A-C]. Data not shown)

Following FACS, total RNA extracted from sorted cells was subjected to Bioanalyzer quality analysis with picogram sensitivity (figure 2.8). Although in some RNA samples the control 18S and 28S ribosomal RNAs could be visualized (e.g. lanes 5 and 7 of figure 2.8A), RNA integrity numbers (RINs) were for the most cases low (example electropherogram in figure 2.8B; high quality RNA considered at RIN>7) when compared to the total mouse brain RNA reference sample (which presented a RIN of 8.90 in combination with a high RNA concentration; lane 8, figure 2.8A; figure 2.8C) (lane 1: RNA at 2,014pg/μl, RIN 1.30; lane 2: RNA at 85pg/μl, RIN 5.60; lane 3: RNA at 131pg/μl, RIN 4.90; lane 4: RNA at 22pg/μl, RIN 4.80; lane 5: RNA at 114pg/μl, RIN 8.70; lane 6: RNA at 22pg/μl, RIN 9.30; lane 7: RNA at 73pg/μl, RIN 6.50; lane 8: RNA at 1,705pg/μl, RIN 8.90).

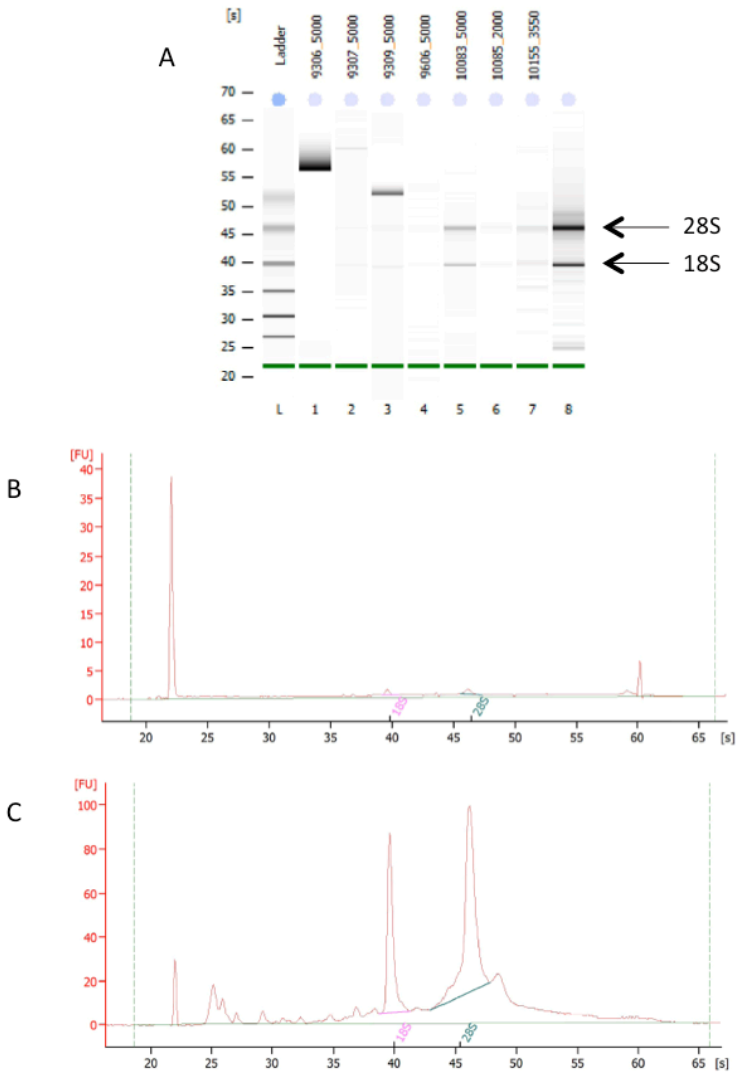


Figure 2.8: **Picogram sensitivity quality analysis of RNA extracted from neurons purified via FACS.** A) Electrophoresis run summary with the reference 18S and 28S ribosomal RNAs indicated by arrows (lane L: RNA ladder; lanes 1-2: YFP D_2-Cre mice; lanes 3-5: ChR D_2-Cre mice; lanes 6-7: ChR D_1-Cre mice; lane 8: total mouse brain RNA reference sample). Electropherograms showing the 18S and 28S peaks within the analysis of B) low quality total RNA (lane 2, RNA integrity number [RIN] 5.60) in comparison to C) high quality total RNA (lane 8, RIN 8.90).

As stated above, we were unable to extract high quality RNA (high RIN and RNA concentration) from FACS-isolated MSNs for further transcriptional analysis. In a further attempt at neuronal isolation of genetically identified circuits, we attempted laser capture microdissection (LCM) as an alternative to FACS. LCM allows for microscope- and laser-assisted dissection of frozen tissue samples, such as brain tissue in our case. The advantage of this method lies in the biochemical integrity of the extracted tissues/cells, as samples are processed still frozen and cellularly intact, unlike FACs where tissue samples are dissociated and individual cells are isolated from cell suspensions (a procedure that may be particularly traumatic for tissues characterized by complex cellular processes and a tight cellular matrix, such as is the case of the brain).

We extracted RNA from sets of 30-50 LCM-isolated YFP^+ neurons and proceeded with microarray analysis against mouse genome GeneChips. Upon analysis of the hybridization data, variation among the different sets of samples resulted in inconsistent results. A tentative list of genes that presented differential expression between the ChR2 and YFP groups was however created. Positive fold-changes (that is, genes with a higher expression rate in $ChR2^+$ cells than in YFP^+ ones) revealed one biologically interesting candidate, Prkcz, with a notable role in neural plasticity (Sacktor et al., 1993) and that, consequently, would constitute a logical target for activity-dependent modulation. This target gene was thus included in the experiments described in the next chapter.

Discussion

In this study, we show that self-stimulation of both striatonigral and striatopallidal DLS neurons is sufficient to positively reinforce actions, but that stimulation of each pathway supports the learning of different action strategies. While animals receiving stimulation in striatonigral neurons acquired the task rapidly, pressed almost exclusively the active lever and were sensitive to changes in contingency, mice self-stimulating striatopallidal neurons acquired lever pressing more slowly (and never pressed as much), generalized pressing from the active to the inactive lever, and were insensitive to contingency degradation. These results suggest that pairing activation of striatonigral neurons in DLS with an action leads to the establishment of a goal-directed relation between that action and the outcome (A-O association), while pairing activation of striatopallidal neurons in DLS with an action supports the formation of a stimulus-response habit that generalizes to similar manipulanda (S-R interaction) (Hilário et al., 2012) and is insensitive to changes in contingency (Yin and Knowlton, 2006).

These conclusions are consistent with the role of long-lasting plasticity of glutamatergic inputs into DLS striatopallidal neurons in both habit formation and skill consolidation (Yin et al., 2009; Yu et al., 2009). This role may be different in DMS, where the striatonigral and striatopallidal pathways have been shown to apparently support opposite roles in reinforcement (Kravitz et al., 2012). These results could also be consistent with a view in which both striatal projection pathways are involved in action selection, with striatonigral neurons supporting the execution of the desired action pattern, and striatopallidal neurons avoiding the execution of competing action patterns (Mink, 1996; Cui et al., 2013); in this view self-stimulation of striatopallidal neurons could mainly support the avoidance of actions other than lever pressing in that particular context. Still, it is clear from these results that in DLS, self-stimulation of striatopallidal neurons is not aversive. In this context, it is interesting to note that optogenetic stimulation of iMSNs (striatopallidal neurons) leads to the activation of a subset of cortical M1 neurons

(Oldenburg et al., 2015), and that inactivation of iMSNs does not necessarily increase basal ganglia output activity (Tecuapetla et al., 2014), underscoring that the functional organization of basal ganglia output is more complex than classically proposed. Lever-pressing activity in both pathways precedes action initiation (Cui et al., 2013), and plasticity associated with instrumental learning could be due to strengthening of corticostriatal synapses that were recently active. Alternatively, stimulation of MSNs can lead, through the cortico-striato-thalamocortical loops, to selection of specific cortical neurons that were previously active.

Taken together, these results show that in DLS both striatonigral and striatopallidal activation can support positive reinforcement of actions paired with that activation, but that the action strategies learned are different. These findings may have implications for understanding the basal ganglia circuitry underlying compulsive actions and persistent habits.

We also took advantage of D1- and D2-mediated expression of ChR2/YFP to attempt the cellular isolation of striatonigral and striatopallidal neurons for transcriptional analysis. FACS was performed on striatal tissue from optogenetically-controlled D1- and D2-YFP/ChR2-YFP mice, but we were unable to extract high quality RNA from FACS-isolated cells. A possible explanation for this might be the extent to which the preparation of cellular material for FACS (resulting in a single cell suspension) compromises the biochemical integrity of the cells being dissociated. By converting cells as asymmetrical as neurons into approximate spheres — capable of passing through the cytometer's flow chamber sheath fluid as a single cell stream — and breaking apart most dendrites and axons, intracellular integrity is being potentially reduced, which could explain the low quality of RNA extracted from FACS-isolated cells. Additionally, the considerable amount of time between the production of neuronal cell suspensions and FACS itself might also explain the extent to which biomolecular integrity may be compromised in such a setup. An alternative technical possibility might be the use of Laser Capture Microdissection (LCM), which has the advantage of not requiring tissue dissociation (as it typically involves immediate freezing of tissue intended for

downstream LCM processing), which keeps cells morphologically undamaged and the intracellular environment of frozen cells biochemically intact. In a recent report, Bandyopadhyay et al. (2014) used LCM to purify mouse spinal cord motor neurons for RNA extraction, with reported RINs of 9.8 and RNA concentrations of 4.9ng/µl (4,900pg/µl) extracted from approximately 4000 cell bodies. The application of similar approaches to optogenetically-controlled neurons, as an extension to our described attempts with this technique, could extend the transcriptomics potential of LCM to circuit neuroscience.

Experimental procedures

Animals. All procedures were reviewed and performed in accordance with the Champalimaud Centre for the Unknown Ethics Committee guidelines, and approved by the Portuguese Veterinary General Board (*Direcção Geral de Veterinária*, approval 0421/000/000/2014). Male mice between 2 and 5 months of age, resulting from the backcrossing of BAC transgenic mice into Black C57BL for at least 8 generations (which express the Cre recombinase under the control of the dopamine D1a (EY217 line) or D2 (ER43 line) receptor promoters) were used in this study. These lines were chosen because their expressions is more restricted to striatum, to avoid possible contaminations from any cortical stimulations. After surgery mice were housed individually under a 12 hours light/dark cycle. Experiments were performed on the light cycle.

Surgery and Histology. Surgeries were performed under anesthesia using a mix of oxygen (1 – 1.5 l/min) and isoflurane (1 – 3 %). Each animal was bilaterally injected with 1.5 μl of viral solution in dorsolateral striatum (DLS – anterior-posterior: 0.38 mm from bregma, mediolateral: 2.5 mm from bregma; dorsoventral: 2.2 mm from the brain surface)[16], using a glass pipette, by pressure (nanojet II from Drummond Scientific, with 4.6 nl pulses at a rate of 0.4 Hz). The viruses injected were AAV2/1.EF1a.DIO.hChR.eYFP (University of North Carolina, titer 5.58 x 10^{12}) for ChR animals, and AAV2/1.EF1a.DIO.eYFP (University of North Carolina, titer 1.85 x 10^{12}) for control animals. For optical stimuli delivery, fiber optics (200 μl diameter, NA=0.22)[17] were implanted at the site of injection, 2.0 mm from the brain surface. Animals were sacrificed after completion of the behavior. Following anesthesia, both control and ChR groups were perfused with saline and paraformaldehyde (4%). Their brains were removed for histological analysis and sectioned in 50 μm coronal slices (Leica vibratome). Both placement of fibers and spread of injection were investigated using a Zeiss AxioImager.M2 widefield fluorescence scanning microscope.

Behavioral procedures. Two weeks after surgery, the behavior of the animals was tested in an instrumental task. Training took place in behavioral chambers (MED-PC, dimensions 23 cm x 20 cm x 19.5 cm – W x D x H) placed in sound attenuating boxes. Each chamber was equipped with a food magazine, a house light place on the wall on the left of the magazine and two retractable levers, one on each side of the magazine. MED-PC IV software was used to control the equipment and record lever presses, head entries to the magazine and laser on-set. Master8 software was used to drive the laser pulses, and Labview was used to record the behavior of the animals in video. Optical stimuli were delivered to both ChR and control animals with implantables fibers[17] connect to a rotatory joint (Doric Lenses) coupled to a 200 mW and 473 nm laser (Shanghai Dream Lasers Technology Co., Ltd). Each stimulus was presented in 10 ms pulses of 5 Hz[18] during 2 seconds, driven by an acousto-optic modulator (AA Opto Electronic) receiving TTL pulses from a Master8 stimulator (A.M.P.I.). The power of the laser was adjusted ex-vivo to be 5-10 mW per hemisphere at the tip of a reference fiber. During training, a session started with the illumination of the house light and extension of both levers. One lever was the active lever (AL) and one was the inactive lever (IL). The levers used as AL and IL were counterbalanced within groups. Optical stimuli to the dorsolateral striatum (DLS) were delivered contingently upon pressing the AL. Animals were trained one session a day, during 30 minutes each and no limit on the number of stimuli, on a continuous reinforcement (CRF) schedule, where each press led to one stimulus. For D_2 animals, animals were trained on CRF for 32 days. D_1 animals were trained for at least 15 days. After acquisition mice received contingency degradation training. In each CD session (30 minutes long) laser onset happened at a random time schedule and non-contingent upon lever press, i.e. independent of the animals' behavior. The number of laser stimulations was yoked independently for each animal, based on their average presses on the last 3 days of training. The D_1 group had 1 session of CD, while the D_2 group had 2 session of CD (to guarantee that indeed they were

not sensitive to CD). Following CD animals received a session of reinstatement, equal to the CRF training sessions before CD.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., CA, USA). Repeated measures ANOVA were used to evaluate acquisition of lever presses and contingency degradation, followed by post hoc analyses using the Dunnet's test and the Sidák correction when appropriate. Statistical significance was set at alpha=0.05. Results were represented as mean ± SEM.

Enzymatic striatal cell dissociation and FACS. Mice subjected to similar AAV2/1 surgical injection, but different optogenetic experiment (placed in a 40cm x 40cm open field arena kept in a sound attenuating box, and subject to 1mW blue laser stimulation at 14Hz for 10s, followed by 50s without laser stimulation, repeated 20 times to a total trial duration of 20 minutes, with one session per each of 5 experimental days), were anesthetized using a mix of oxygen (1–1.5 l/min) and isoflurane (1–3%), sacrificed by cervical dislocation, their brains quickly removed and transferred to ice-cold phosphate buffered saline (PBS). Total striatum was dissected from both hemispheres and dissociated for 45 minutes at 37ºC with Papain enzyme (Papain Dissociation System, Worthington Biochem) in Earle's Balanced Salt Solution (EBSS) with DNase according to the manufacturer's indications. Dissociated striata were triturated with two glass pipettes of decreasing tip diameter, the suspension containing dissociated cells was transferred to a different falcon tube and then centrifuged at 900RPM for 5 minutes at room temperature (RT). To remove excess debris, cell pellets were resuspended in a mix of EBSS, DNase and albumin ovomucoid inhibitor (AOI, according to the Worthington Biochem Papain Dissociation System specifications), and re-centrifuged on an AOI discontinuous gradient at 900 RPM at 4ºC for 6 minutes. Cell pellets were resuspended in buffer media (L15-CO_2 without phenol, 1x Pen-Strep, 10 mM Hepes, 25 μg/ml DNase, 1mg/ml BSA) and filtered through a 70μm

mesh (BD Falcon #352350). Cells were then sorted on a FACSAria III cell sorter (BD Biosciences) for FITC signal (exciting YFP at 488nm and detecting the signal with a 530/30 nm filter) with a 70μm nozzle and 70psi of pressure. Approximately 1000-2000 YFP^+ neurons were FACS sorted per experimental animal. Cells were kept on ice pre- and post-sorting. Total RNA from YFP^+ sorted cells was extracted using the PicoPure RNA isolation kit (ThermoFisher Scientific), according to the manufacturer's indications. RNA quality was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies) and RIN determined by electropherogram analysis.

References

Albin RL, Young AB and Penney JB (1989) **The functional anatomy of basal ganglia disorders**. Trends in Neuroscience 12: 366-375.

Bandyopadhyay U, Fenton WA, Horwich AL and Nagy M (2014) **Production of RNA for transcriptomic analysis from mouse spinal cord motor neuron cell bodies by laser capture microdissection**. Journal of Visualized Experiments 13:e51168.

BD Biosciences (2000) **Introduction to Flow Cytometry: a learning guide**. BD Biosciences, San Jose, California, USA: Manual Part Number: 11-11032-01.

Cui G, Jun SB, Jin X, Pham MD, Vogel SS, Lovinger DM and Costa RM (2013) **Concurrent activation of striatal direct and indirect pathways during action initiation**. Nature 494: 238-242.

DeLong MR (1990) **Primate models of movement disorders of basal ganglia origin**. Trends in Neuroscience 13: 281-285.

Durieux PF, Schiffmann SN and de Kerchove d'Exaerde A (2011) **Targeting neuronal populations of the striatum**. Frontiers in Neuroanatomy 5: 1-9.

Ena S, de Kerchove d'Exaerde A and Schiffmann SN (2011) **Unraveling the differential functions and regulation of striatal neuron sub-populations in motor control, reward, and motivational processes**. Frontiers in Behavioral Neuroscience 5: 1-10.

Frank MJ, Seeberger LC and O'Reilly RC (2004) **By carrot or by stick: cognitive reinforcement learning in parkinsonism**. Science 306: 1940-1943.

Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ Jr. and Sibley DR (1990) **D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons**. Science 250: 1429-1432.

Gong S, Doughty M, Harbaugh CR, Cummins A, Hatten ME, Heintz N and Gerfen CR (2007) **Targeting Cre recombinase to specific neuron populations with bacterial artificial chromosome constructs**. Journal of Neuroscience 27: 9817-9823.

Hilário MRF, Clouse E, Yin HH and Costa RM (2007) **Endocannabinoid signaling is critical for habit formation**. Frontiers in Integrative Neuroscience 1: 1-12.

Hilário MRF, Holloway T, Jin X and Costa RM (2012) **Different dorsal striatum circuits mediate action discrimination and action generalization**. European Journal of Neuroscience 35: 1105-1114.

Jin X, Tecuapetla F and Costa RM (2014) **Basal ganglia subcircuits distinctively encode the parsing and concatenation of action sequences**. Nature Neuroscience 17: 423-430.

Kravitz AV, Tye LD and Kreitzer AC (2012) **Distinct roles for direct and indirect pathway striatal neurons in reinforcement**. Nature Neuroscience 15: 816-818

Lobo MK, Karsten SL, Gray M, Geschwind DH and Yang XW (2006) **FACS-array profiling of striatal projection neuron subtypes in juvenile and adult mouse brains**. Nature Neuroscience 9: 443:452.

Mink JW (1996) **The basal ganglia: focused selection and inhibition of competing motor programs**. Progress in Neurobiology 50: 381-425.

Okaty BW, Sugino K and Nelson SB (2011) **Cell type-specific transcriptomics in the brain**. Journal of Neuroscience 31: 6939-6943.

Oldenburg IAA and Sabatini BLL (2015) **Antagonistic but not symmetric regulation of primary motor cortex by basal ganglia direct and indirect pathways**. Neuron 86: 1174-1181.

Sacktor TC, Osten P, Valsamis H, Jiang X, Naik MU and Sublette E (1993) **Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation**. Proceedings of the National Academy of Sciences USA 90: 8342:8346.

Tecuapetla F, Matias S, Dugue GP, Mainen ZF and Costa RM (2014) **Balanced activity in basal ganglia projection pathways is critical for contraversive movements**. Nature Communications 5: 4315.

Yin HH and Knowlton BJ (2006) **The role of the basal ganglia in habit formation**. Nature Reviews Neuroscience 7: 464–476.

Yin HH, Mulcare SP, Hilário MRF, Clouse E, Holloway T, Davis MI, Hansson AC, Lovinger DM and Costa RM (2009) **Dynamic reorganization of striatal circuits during the acquisition and consolidation of a skill**. Nature Neuroscience 12: 333-341.

Yu C, Gupta J, Chen J and Yin HH (2009) **Genetic deletion of A_{2A} adenosine receptors in the striatum selectively impairs habit formation**. Journal of Neuroscience 29: 15100-15103.

"We can't any longer have the conventional understanding of genetics which everybody peddles because it is increasingly obvious that epigenetics - actually things which influence the genome's function - are much more important than we realised."

Robert Winston

Chapter III

RNA Pol II phosphorylation dynamics in the striatum during skill learning

Manuscript in preparation: Galvão-Ferreira P, Lipinski M, Barco A and Costa RM. **RNA Pol II RPB1 phosphorylation is modulated by motor skill learning in the mouse brain**.

Author contributions: Pedro Galvão-Ferreira, Michał Lipinski, Angel Barco and Rui M Costa designed the experiments. Pedro Galvão-Ferreira and Michał Lipinski performed the experiments. Pedro Galvão-Ferreira and Michał Lipinski analyzed the data.

Abstract

A multi-layered complexity of epigenetic regulatory mechanisms vastly underlies activity-dependent neuronal transcription, from the biochemical modification of chromatin proteins and DNA CpG cytosines, to modulation of the transcription machinery itself. In the last two decades, the regulation of RNA Pol II progression along the transcription cycle has been shown to be a major factor in transcriptional regulation. The balance between promoter-proximal pausing and elongating RNA Pol II — sustained by the regulated phosphorylation of serine residues in the heptapeptide consensus sequence YSPTSPS on the CTD of RPB1, RNA Pol II's largest subunit — has been observed in several organisms, tissues and developmental stages, including the adult mouse nervous system, but has never been shown to be modulated by learning. Here, we investigated the impact of learning a motor skill on RNA Pol II phosphorylation dynamics in the mouse striatum. We show that learning a motor skill modulates the *in vivo* striatal phosphorylation dynamics of RNA Pol II RPB1, a decrease of striatal levels of Ser2P^{+}-enriched RPB1 and an increase in the pausing index in trained mice. We also demonstrate that this modulation occurs at the level of immediate early genes (IEGs), with both Arc and c-Fos showing an increased pausing index of RNA Pol II bound to the promoter and the gene sequence. Our experiments provide, to the best of our knowledge, the first demonstration of RNA Pol II pausing in the adult brain in the context of learning.

Introduction

The nervous system is the mediator between an animal and the surrounding environment (reviewed in Wolf and Linden, 2012). This interaction with an environment is conveyed through changes in neuronal connectivity, structure and activity that mold neural circuits in an activity-dependent manner for short- or long-lasting changes (reviewed in West and Greenberg, 2011; Lyons and West, 2011). Long-lasting consolidation of skills requires neuronal adaptability in different brain systems at different levels, and it may include adjustments to the transcription of neuronal genomes. As an extremely complex and multi-dimensional structure liable to dynamic compaction and opening, chromatin contains those genomes and regulates their transcription (reviewed in Hager et al., 2009; Levine et al., 2014). Many epigenetic mechanisms, from acetylation and methylation of histones to cytosine DNA methylation, have a comprehensive impact on gene expression, because they help orchestrate the harmonious sequence of chromatin remodeling and effective transcriptional regulation; many of these epigenetic regulatory mechanisms mediate neuroplasticity by linking the activity of chromatin remodeling enzymes (such as histone deacetylases [HDACs]), Ca^{2+}-dependent signaling proteins and activity-dependent transcription factors (reviewed in Hager et al., 2009; Meaney and Ferguson-Smith, 2010; Wolf and Linden, 2012; Levine et al., 2014).

Transcription itself may be regulated at multiple stages. One of the possible checkpoints is the progression of RNA Pol II throughout the transcription cycle by phosphorylation of the serine residues along the heptapeptide consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser ($Y_1S_2P_3T_4S_5P_6S_7$) at the carboxy terminal domain (CTD) of its largest subunit, RPB1, with an enrichment of $Ser5P^+$ RNA Pol II around the transcription start site, and increase of $Ser2P^+$ residues in actively-transcribing RNA Pol II (reviewed in Jonkers and Lis; 2015). First identified in *Drosophila melanogaster* heat shock protein (Hsp) genes (Gilmour and Lis, 1986; Rougvie and Lis, 1988; Rougvie and Lis, 1990; Rasmussen and Lis,

1993), this ability of RNA Pol II to pause in promoter-proximal regions is also present in neurons of the central nervous system, where it has been shown to regulate the activity-dependent transcriptional dynamics of immediate early genes (IEGs) (Saha et al., 2011). However, this mechanism has not been studied in the adult, *in vivo*, brain in the context of learning. With this in mind, we set out to explore the impact of learning a motor skill on RNA Pol II pausing in the mouse striatum. Using a fast lever-pressing task as a motor skill-learning paradigm, we examined the global phosphorylation dynamics of RNA Pol II, and then profiled the binding kinetics of the different RPB1 phospho-variants to the promoters and gene bodies of IEGs. These experiments provide the first example of RNA Pol II phosphorylation modulation in the brain in the context of learning.

Results

3.1 Fast lever-pressing operant training

To examine the impact of learning a motor skill on RNA Pol II RPB1 phosphorylation dynamics, we trained animals in a fast lever-pressing operant task. In this task, animals were first taught to associate pressing a lever with receiving a food pellet in a continuous reinforcement schedule (CRF), with one lever press resulting in delivery of one food pellet to the magazine, to a maximum of 30 pellets per session. After CRF, animals were asked to perform eight lever presses to receive one food pellet (i.e. with a fixed ration of eight lever presses per food pellet; FR8), but having to do so within a time contingency: FR8-1000s (i.e. eight lever presses within 1000 seconds), FR8-500s, FR8-50s, FR8-10s, FR8-5s, FR8-4s, FR8-3s, FR8-2s and FR8-1s, with animals finishing their fast lever-pressing training pressing the lever at 8Hz.

Mice consistently showed a steady and continuous increase in the average number of lever presses per session (figure 3.1A). This tendency for an escalation in lever-pressing is explained by the increasing difficulty in the training regime, as sessions progress towards decreasing time contingencies in which to perform the sequences of eight lever presses. An analysis of the performance of the sequences across training demonstrates that mice displayed gradually reduced inter-press intervals (IPIs; figure 3.1B) and a decreasing distance to the final target of 150ms (as the optimized IPI at FR8-1s: 7 IPIs of approximately 150 milliseconds each; figure 3.1C). These data indicate that mice learned to preform this motor skill, which is dependent on striatal plasticity (Jin 2010, Jin 2014, Santos 2015).

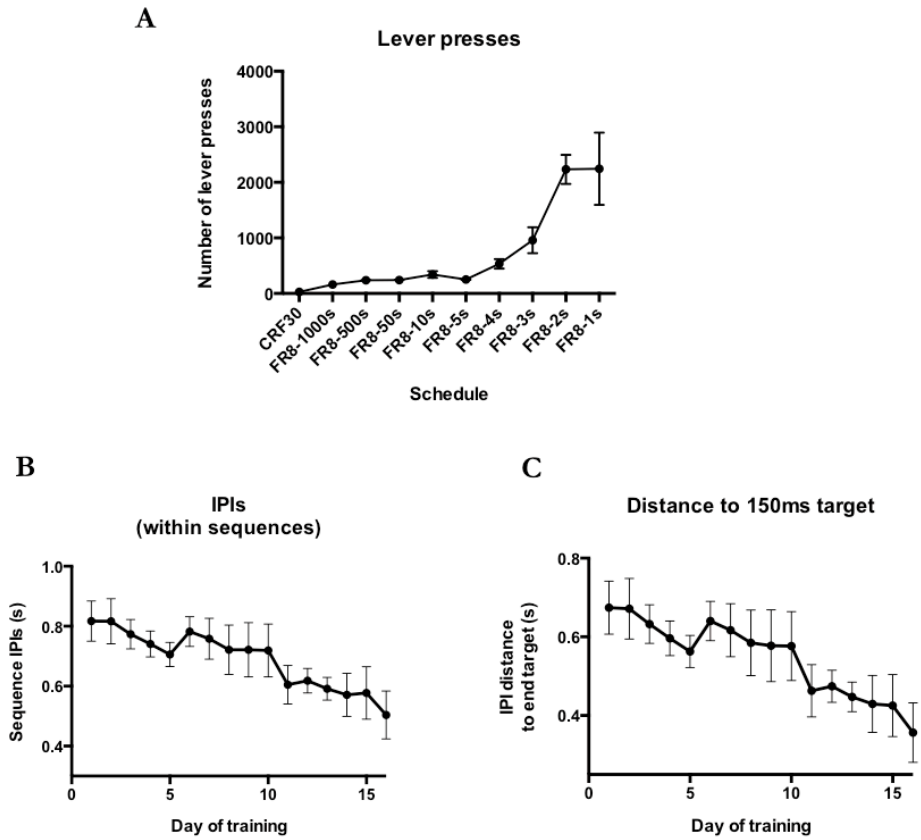


Figure 3.1: **Animal performance during a fast lever-pressing task.** After one session of continuous reinforcement with self-paced delivery of up to thirty food pellets (CRF30), animals (n=7) were required to perform on a fixed ratio schedule, whereby eight lever presses resulted in delivery of a food pellet within a time contingency, which ranged from one-thousand to one second (FR8-1000s to FR8-1s). A) Acquisition of the task as represented by the average number of lever presses in each day of training. B) Inter-press intervals (IPIs) within defined sequences ($F_{2.942,17.65} = 5.136$, P=0.0102). C) Distance of all 7 consecutive IPIs from the final covert target ($F_{2.823,16.94} = 5.423$, P=0.0093). Mean ± SEM represented in all graphs.

3.2 RNA Pol II RPB1 striatal phosphorylation in mice trained in a fast lever-pressing task

To test if motor skill learning had an impact on striatal levels of RNA Pol II RPB1 CTD phosphorylation, we assayed total protein extracts from the striatum of mice trained in the fast lever-pressing task (n=7) and those naïve to the operant task (i.e. control mice; n=7) with an antibody that recognizes total RPB1 CTD regardless of the specific phosphorylated residues (figure 3.2).

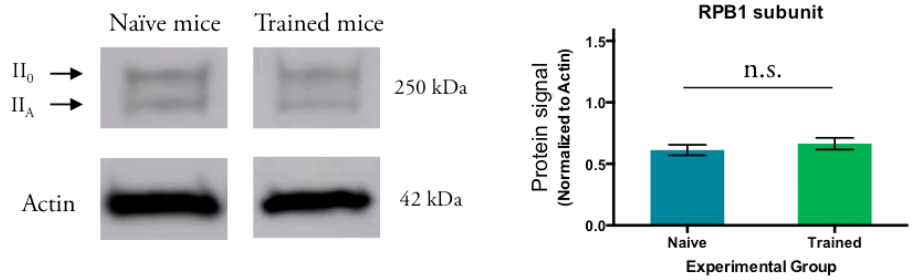


Figure 3.2: **RNA Polymerase II total RPB1 phosphorylation in the striatum of mice trained in the fast lever-pressing task.** Immunoblot analysis of the total RPB1 CTD repeat $YS_2PTS_5PS_7$, with indication of its hyperphosphorylated (II_0) and hypophosphorylated (II_A) forms. Change fold data as mean ± SEM; n.s., $P > 0.05$.

Due to the differences in the relative amount of phosphorylation, protein extracts probed with an anti-RPB1 CTD antibody resolve in two different bands around 250kDa: that corresponding to the hyperphosphorylated (II_0), and hence heavier, form of the RPB1 CTD, and the lighter hypophosphorylated (II_A) form. We observed no significant differences in the global levels of RPB1 CTD across trained and control mice. This is not surprising, as we did not expect learning to induce a manifest difference in the total amount of the RNA Pol II protein but, rather a modulation of the phosphorylation levels within the pool of existing RNA Pol II molecules.

3.3 RNA Pol II RPB1 Ser5P$^+$ and Ser2P$^+$ striatal phosphorylation in mice trained in a fast lever-pressing task

Next, we asked whether we would observe a modulation of the phosphorylation levels of RNA Pol II RPB1 CTD as a result of mice undergoing the motor skill-learning paradigm (figure 3.3).

As RNA Pol II molecules elongate towards productive transcription and the balance between Ser5P$^+$- and Ser2P$^+$-enriched RPB1 CTD changes, with RNA Pol II being released from the promoter-proximal paused state by the P-TEFb complex, the RPB1 CTD decreases in Ser5P$^+$ and increases in Ser2P$^+$ (reviewed in Jonkers and Lis; 2015). Here, we did not observe a significant difference in the levels of RPB1 Ser5P$^+$-enriched CTD between control and trained mice (figure 3.3A). However, when we examined the levels of Ser2P$^+$-enriched CTD, we observed a marked decrease of signal in trained animals when compared with untrained naïve controls exposed to the box (figure 3.3B).

To test if these differences in RPB1 phosphorylation were due to fluctuations in the global transcriptional levels in the striatum as a result of training (figure 3.3C), we compared the actin levels between control and naïve mice, finding no statistically significant differences between them.

A common measure, or indicator, of RPB1 phosphorylation dynamics is the pausing — or "poising" — index, calculated as the RPB1 Ser5P$^+$ signal divided by the RPB1 Ser2P$^+$ signal. This index functions as a proxy for the balance between promoter-proximal-bound RNA Pol II molecules, and those that are involved in productive elongation in downstream gene regions. When we calculated the pausing index for our trained and control mice, we found a robust difference between these two groups of mice, with an increase in the poising index of trained mice when compared with their naïve littermates (figure 3.3D).

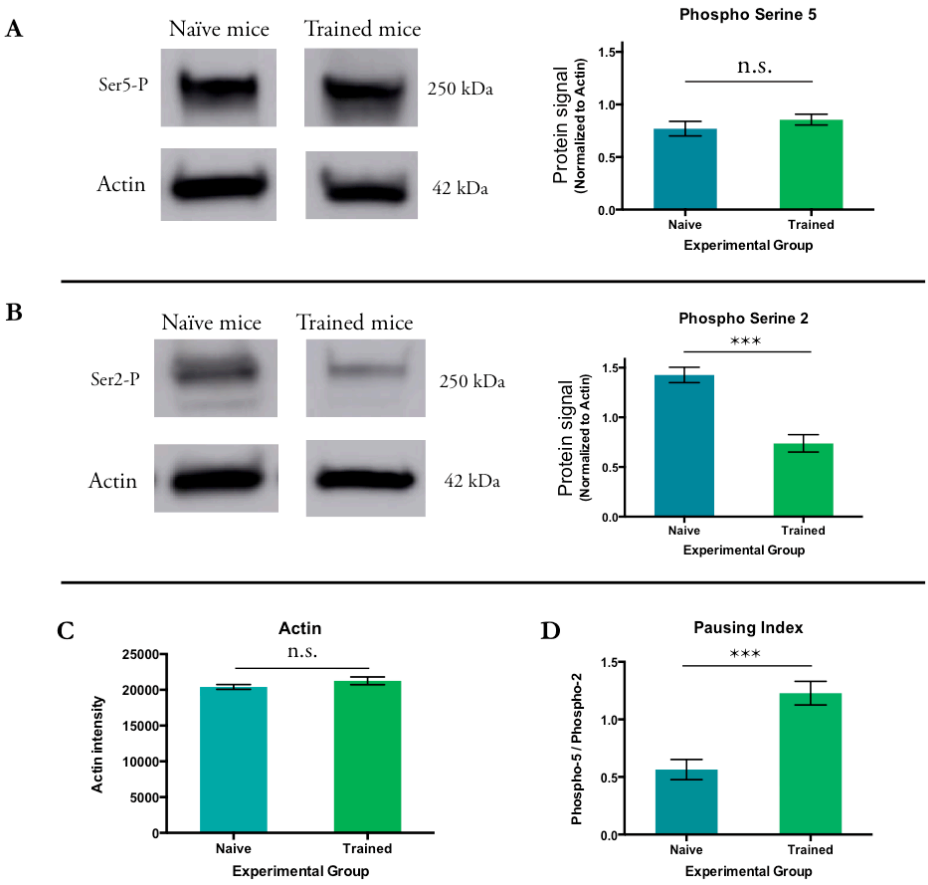


Figure 3.3: **RNA Polymerase II RPB1 CTD phosphorylation forms in the striatum of mice trained in the fast lever-pressing task.** Immunoblot analysis of the RPB1 CTD repeat $YS_2PTS_5PS_7$ positive for the phospho-isoforms A) Ser5-P or B) Ser2-P. C) Actin quantification across both phospho-isoforms. D) RNA Polymerase II pausing index (calculated as the quotient between the Ser5-P and Ser2-P RPB1 CTD phospho-isoforms) in the striatum of mice trained in the fast lever-pressing. Change fold data as mean ± SEM; ***, $P < 0.005$; n.s., $P > 0.05$.

3.4 RNA Pol II RPB1 Ser5P$^+$ and Ser2P$^+$ striatal phosphorylation in two different groups of control mice

When we first submitted mice to the fast lever-pressing task, the protein signals of trained mice was compared to that of control littermates exposed to similar behavioural boxes. We did not, however, supply these control animals with the same amount of food pellets their trained littermates had received as a result of the training paradigm. As a result, we sought to rule out the possibility of the phosphorylation differences we found between trained and the initial behavioural box exposure control (or "context control") animals being exclusively due to the absence of the "reward" food pellets during training sessions (as all naïve and trained groups were food deprived, but the initial "context controls" were not fed the same number of pellets the trained groups received as reinforcers), and not to learning of the motor skill itself.

Consequently, we produced a new control cohort, which we dubbed "context+reward" (figure 3.4), in which mice were exposed to the same behavioural boxes as trained mice, but received approximately 30 pellets as a result of the exposure session so as to mimic a food pellet reward similar to that received by trained animals. When we compared the levels of Ser5P$^+$- (figure 3.4A) and Ser2P$^+$-enriched RPB1 CTD (figure 3.4B) in "context control" and "context+reward" animals, we found no significant differences between these two groups for either phosphorylation form. Therefore, as expected, the calculated pausing index between these two groups was shown to be equally similar (figure 3.4C).

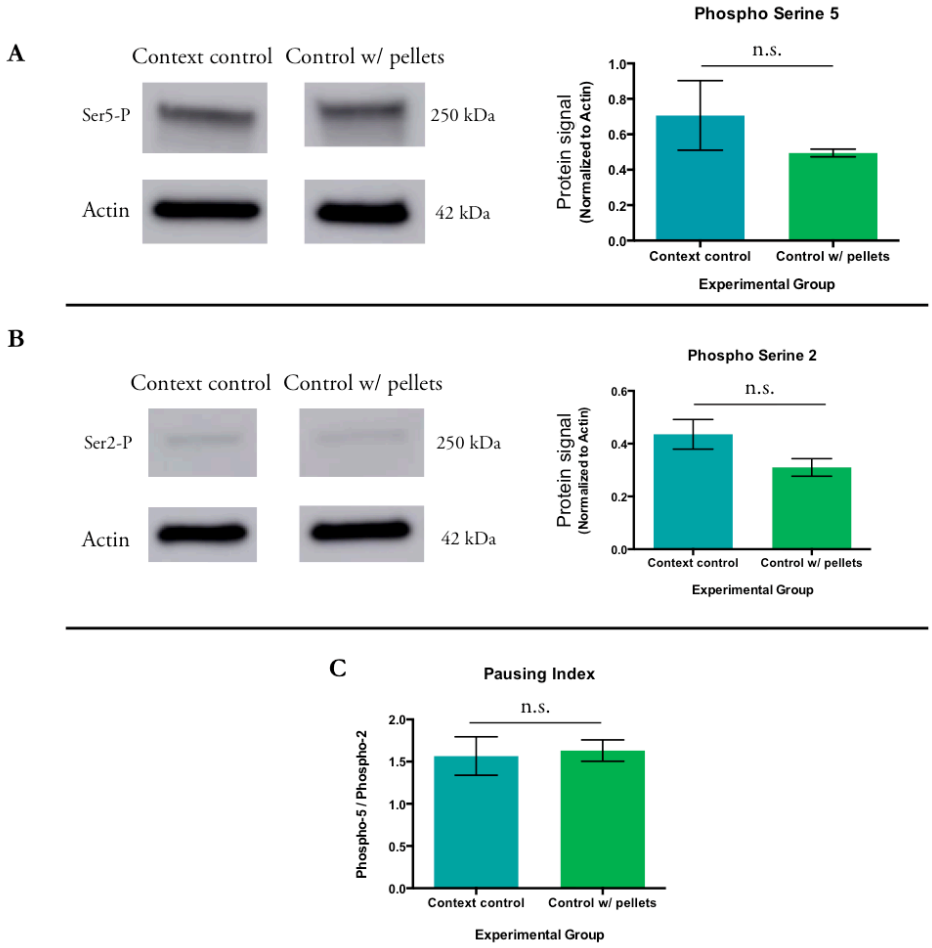


Figure 3.4: **RNA Polymerase II RPB1 CTD phosphorylation forms in the striatum of two different groups of control mice for the fast lever-pressing task.** Immunoblot analysis of the RPB1 CTD repeat $YS_2PTS_5PS_7$ positive for the phospho-isoforms A) Ser5-P or B) Ser2-P, as well as C) the RNA Polymerase II pausing index in "context control" and "context+reward" (control with pellets) animals. Change fold data as mean ± SEM; n.s., $P > 0.05$.

3.5 RNA Polymerase II RPB1 CTD binding to target genes in the striatum of mice trained in a fast lever-pressing task

In their seminal paper on neuronal activity-regulated modulation of RNA Pol II poising, Saha et al. (2011) demonstrated that the onset of neuronal activity triggered a displacement of Ser5P^{+}-rich RNA Pol II molecules from gene promoters into actively transcribing Ser2P^{+}-rich isoforms. They also showed that priming of IEGs (genes that are rapidly and transiently activated in response to neuronal activity, such as Arc and c-Fos [Pérez-Cadahía et al., 2011]) by poised RNA Pol II was, at least partly, responsible for their fast induction kinetics upon neuronal activity.

In our study, to investigate if the observed training-induced modulation of RNA Pol II CTD phosphorylation was present at IEGs, we performed chromatin immunoprecipitation followed by quantitative real-time PCR (ChIP-qPCR) on whole striata dissected from mice naïve and trained in the lever-pressing task presented above (figure 3.1).

An investigation of total RPB1 binding to the promoters and gene bodies of Gapdh (a positive control, housekeeping gene), Arc and c-Fos (two paradigmatic IEGs, also examined by Saha et al. [2011]), as well as Prkcz (a gene with a documented role in long-term memory formation; Sacktor et al., 1993) and the Gastrulation Brain Homeobox 2 (Gbx2) gene (a gene involved in brain development [Wassarman et al., 1997] with no significant adult expression; here, together with an intergenic region, used as a negative control) revealed no statistically significant binding differences between naïve and trained mice for any of the targets, but revealed an expected higher presence of RNA Pol II molecules in the promoters of genes relative to their gene bodies (figure 3.5A).

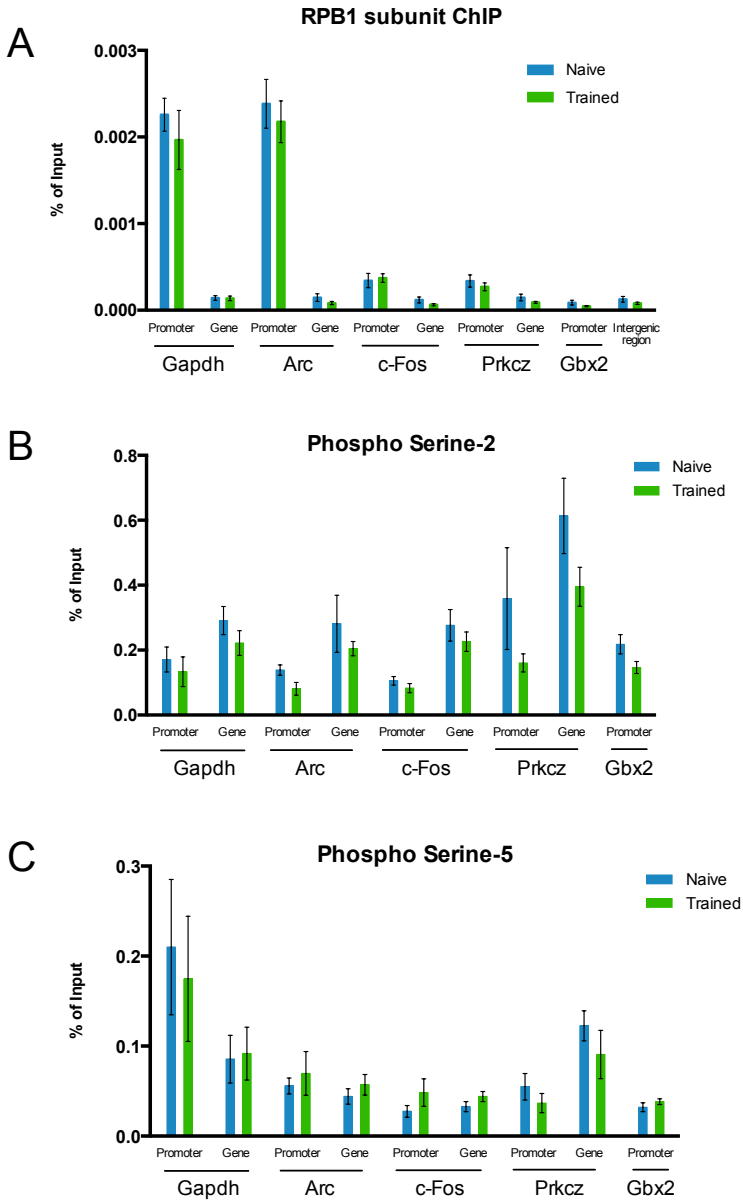


Figure 3.5: **Enrichment of RNA Polymerase II RPB1 CTD phosphorylation forms in target genes in the striatum of mice trained in a fast lever-pressing task.** ChIP-qPCR analysis of A) the total RPB1 CTD repeat $YS_2PTS_5PS_7$, and the RPB1 CTD positive for the phospho-isoforms B) Ser2-P or C) Ser5-P. % of Input data as mean ± SEM.

An examination of RPB1 phospho-variant binding, be it Ser5P$^+$- or Ser2P$^+$-RPB1, to the promoters and gene bodies of the chosen targets, again reveals an absence of statistically significant differences between naïve and trained mice, but seems to present an apparent trend: a decrease in Ser2P$^+$-RPB1 (figure 3.5B), consistent with the decrease observed in the Western blotting experiments above (figure 3.3B), and a potential, lesser increase in Ser5P$^+$-RPB1 with training (figure 3.5C).

3.6 Promoter/gene binding ratios for RNA Polymerase II RPB1 CTD in the striatum of mice trained in a fast lever-pressing task

Next, we analyzed the relation between promoter- and gene body RPB1-binding for each target in naïve and trained mice. We found no statistically significant differences between naïve and trained total RPB1 binding in individual targets, but when all four promoters and gene bodies were taken together and matched as a whole, a statistically significant increase emerged between the naïve and trained groups (figure 3.6A). This difference could signify a displacement of RNA Pol II to gene promoters as a function of training, which would be consistent with an increase in promoter-proximal RNA Pol II pausing. A similar, and slightly more significant, increase in promoter/gene ratio was observed when the two IEGs (Arc and c-Fos) were analyzed together; this effect disappeared when Gapdh and Prkcz were grouped (presenting a nearly identical naïve and trained promoter/gene ratios), suggesting a possible leverage effect by these — and possibly other — IEGs, that shift the ratio of the available RNA Pol II pool towards gene promoters. These naïve to trained differences are not present in any target in the Ser2P$^+$- (figure 3.6B) and Ser5P$^+$-RPB1 (figure 3.6C) promoter/gene ratio analyses.

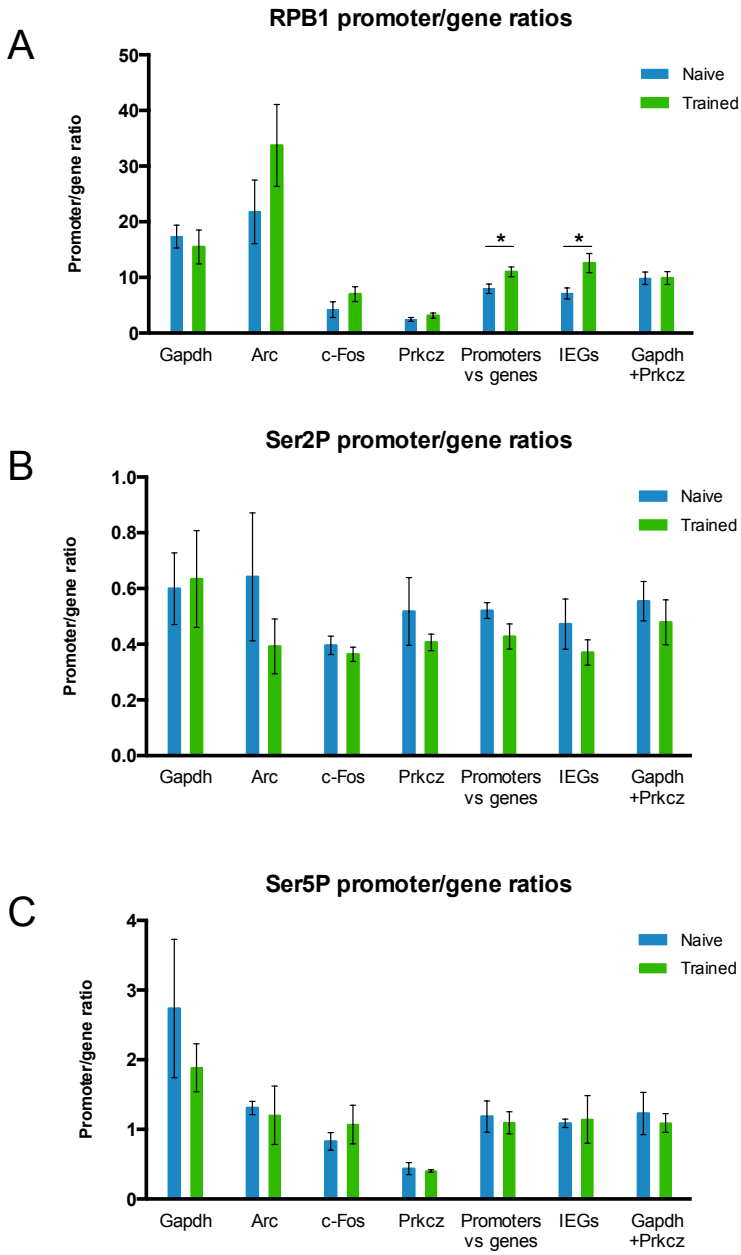


Figure 3.6: **Promoter/gene ratios for RNA Polymerase II RPB1 CTD phosphorylation forms in target genes in the striatum of mice trained in a fast lever-pressing task.** ChIP-qPCR % of input data as a ratio between the promoter and gene bodies of target genes for A) the total RPB1 CTD repeat, and B) the RPB1 phospho-Ser2 CTD or C) the phospho-Ser5 CTD. Promoter/gene ratio data as mean ± SEM; *, $P < 0.05$.

3.7 Ser5P^{+} versus Ser2P^{+} RNA Polymerase II RPB1 CTD in the striatum of mice trained in a fast lever-pressing task

Lastly, we compared the Ser5P^{+}- and Ser2P^{+}-RPB1 levels in naïve and trained mice for all target genes (figure 3.7). We observed a beautiful pattern of Ser5P^{+}- and Ser2P^{+}-RPB1 stabilization with training (a clear difference between naïve and trained animals that disappeared completely), but only at IEG promoters. This Ser5P^{+}- and Ser2P^{+}-RPB1 equilibrium seems to be perfectly reversed at the gene body of Arc (for c-Fos it seems to be at least maintained). This training-induced modulation of Ser5P^{+}- and Ser2P^{+}-RPB1-binding does not appear in any other target, be it promoter or gene body, as the balance between Ser2P and Ser5P in the naïve and trained groups remains stable.

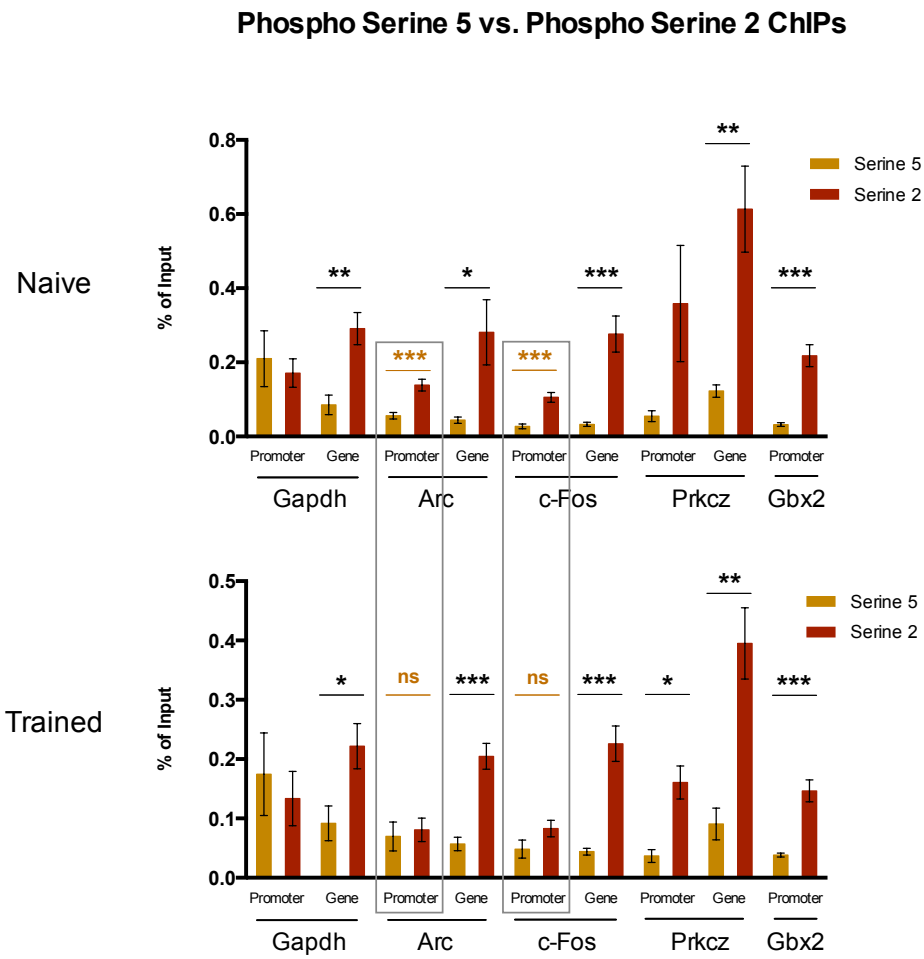


Figure 3.7: **Ser5P$^+$ and Ser2P$^+$ RNA Polymerase II RPB1 CTD in target genes in the striatum of mice trained in a fast lever-pressing task.** ChIP-qPCR % of input data for the Ser5P$^+$ and Ser2P$^+$ RPB1 CTD as a ratio between these two phospho isoforms for target genes. % of Input data as mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.; n.s., $P > 0.05$.

3.8 Ser5P/Ser2P RNA Polymerase II RPB1 CTD ratios in the striatum of mice trained in a fast lever-pressing task

Next, we examined the Ser5P/Ser2P binding ratios (pausing index) for the different target genes. While the Ser5P/Ser2P ratios for both Gapdh and Prkcz presented no statistically significant difference between naïve and trained mice, we did observe an increase in the pausing indexes for the promoters and gene bodies of both IEGs, Arc and c-Fos (figure 3.8). This increase in the pausing indexes of trained mice, here detected specifically for IEGs, is consistent with our Western blotting results, which demonstrated a very clear positive modulation of the Ser5P/Ser2P ratios with training (figure 3.3D). A statistically significant difference between the naïve and trained pausing indexes of the Gbx2 promoter was also found (an observation that will merit further consideration during the discussion at the end of this chapter).

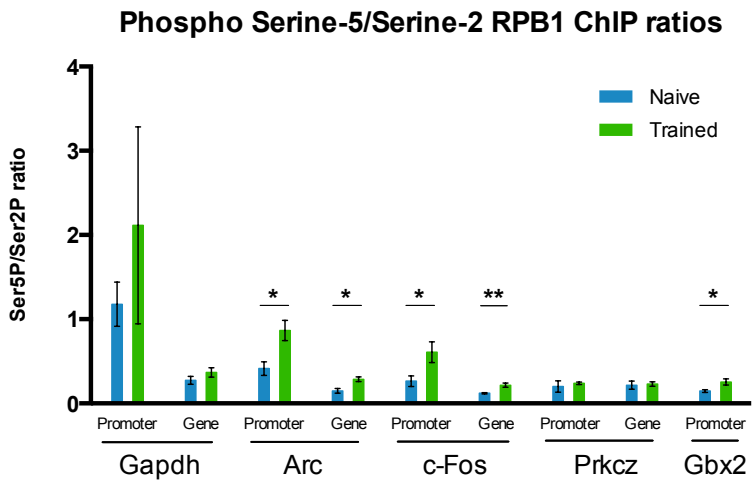


Figure 3.8: **Ser5P/Ser2P RNA Polymerase II RPB1 CTD ratios in target genes in the striatum of mice trained in a fast lever-pressing task.** ChIP-qPCR % of input data for the Ser5P$^+$ and Ser2P$^+$ RPB1 CTD as a ratio between these two phospho isoforms for each target gene. Ser5P/Ser2P ratio data as mean ± SEM; *, $P < 0.05$; **, $P < 0.01$.

Discussion

In this chapter, we asked whether learning a motor skill modulated the mechanism known as RNA Pol II pausing, in which progression of RNA Pol II molecules along the transcription cycle is modulated by dynamic phosphorylation of specific serine residues in the CTD of the RPB1 subunit of RNA Pol II ($YS_2PTS_5PS_7$), with Ser5P$^+$-enriched RPB1 CTD correlated with promoter-proximal-bound RNA Pol II, and Ser2P$^+$-enriched RPB1 CTD associated with more downstream, actively transcribing RNA Pol II.

To answer this question, we first developed a motor training task in which mice were asked to press a lever in order to receive a food reward. This association led to a rapid and solid increase in lever pressing per training session, and to performance of sequences of eight presses within a progressively smaller time, resulting in animals getting closer to the goal of fast lever-pressing at 8Hz for 1s. Once this motor skill was acquired, total protein from the striata of mice trained in the lever-pressing task was probed with antibodies recognizing the RPB1 CTD regardless of phosphorylation status, as well as Ser5P$^+$- or Ser2P$^+$-enriched RPB1 CTD; the same Western blotting experiments were performed using total protein from mice naïve to the motor training task (i.e. control mice which were exposed to the behavioural boxes simultaneously to trained mice, but did not learn the motor skill). With our Western blotting experiments, we made three observations. First, we found no differences in the total levels of RPB1 CTD between naive and trained, which is not surprising, given that a modulation of RNA Pol II pausing-regulated transcriptional programs would more likely involve a dynamic shift in the balance of the specific RPB1 CTD residues being phosphorylated, rather than a change in the amount of RNA Pol II molecules in neurons. Second, we observed constant levels of Ser5P$^+$-enriched RPB1 CTD between naïve and trained mice; however, when we probed total striatal protein of naïve and trained mice for Ser2P$^+$-enriched RPB1 CTD, we found a very robust decrease of RPB1 rich in this phosphorylated serine residue. As a consequence of this difference in Ser2P$^+$

RPB1, the poising index for trained mice is remarkably different from that of naïve mice. This could mean that the available pool of RNA Pol II molecules is shifting from being engaged in active transcription, i.e. elongation, and rather mobilizing itself at the level of gene promoters to facilitate fast transcription as a response to neuronal activity, a shift that could constitute a true molecular hallmark of learning. Third, we tested whether the modulations we observed in RPB1 phosphorylation were due to the reward value of the food pellets trained mice received as part of their skill training, and not to consolidating the motor skill itself, as our initial control group did not received the same amount of food pellets during their exposure sessions as trained mice did during training sessions (as previously mentioned, all naïve and trained groups were food deprived, but the initial "context controls" were not fed the same number of pellets the trained groups received as reinforcers). Consequently, we probed total striatal protein from a second control group (fed the same amount of food pellets as trained mice) for the same protein, and protein phosphoisoforms, as the initial control group, and found no significant differences for either antibody signal between the two control groups.

When we then examined the binding of RPB1 to IEGs by ChIP, we found a modulation of RNA Pol binding at the promoters of these genes, concomitant with the decrease in Ser2P^{+}-enriched RPB1 we observed at the protein level. This shift of pausing indexes at IEG promoters is consistent with the findings of Saha et al. (2011), who had already observed that RNA Pol II pausing resulted in a kinetic advantage to the transcription of rapidly-induced IEGs, including Arc and c-Fos. This seminal study had already demonstrated that the onset on neuronal activity could induce the recruitment of PTEF-b to IEGs, promoting the subsequent activity-dependent phosphorylation of RPB1's Ser2 CTD, releasing RNA Pol II molecules from a promoter-bound state and allowing them to transition to active elongation. A shift towards increasing pausing indexes in mice subjected to learning paradigms was also observed in our ChIP experiments at IEGs, which could lead to and promote the consolidation of learning by fine-tuning gene responses in a precisely timed manner as a response to neuronal activity. This modulation will

very likely be differentially expressed in diverse neural circuits, as a response to different instances of learning, sustained by different neural circuits.

We also found a statistically significant difference between naïve and trained RNA Pol II pausing levels at the Gbx2 promoter. The gene body of Gbx2, however, was not assayed for Ser2P^{+}- or Ser5P^{+}-RPB1 enrichment; this region of the Gbx2 gene, rather than the promoter, should possess much less bound RNA Pol II as would be predicted of a developmentally silent gene and thus function more properly as a negative control. Besides its role in regulating the formation of the midbrain-hindbrain compartments (Wassarman et al., 1997), Gbx2 is also a master regulator of striatal cholinergic interneuron development, a function it exerts through the sequential spatio-temporal developmental specification of cholinergic interneuron populations in the medial and lateral sub-regions of the striatum (Chen et al., 2010). These spatio-temporal expression patterns may be the result of Gbx2's complex regulation by different enhancer regions, the organization of which is conserved among vertebrates (Islam et al., 2006); the combinatorial interaction of enhancers and the transcription machinery has a well known role in regulating developmental timing (reviewed in Lenhard et al., 2012), and may be extended, in the case of Gbx2 enhancers, to still uncharted transcriptional regulatory functions in adult neurons. Striatal cholinergic cells provide the striatum with its principal source of acetylcholine (a neurotransmitter with neuromodulatory properties) (reviewed in Lim et al., 2014), and have been involved in an animal's ability to shift between different action strategies as a form of behavioral flexibility (Aoki et al., 2015) or as being required for dorsomedial (but not dorsolateral) striatum-specific learning of place discrimination and adaptation to reward contingency (Okada et al., 2014). The impact of Gbx2 in striatal physiology might be marginal, but one that may be potentially mapped from development onto adulthood (where a striatal mediolateral involvement of Gbx2 in interneuron developmental specification curiously maps onto the mediolateral role of this cell-type in behavioural flexibility) through an instance of functional redundancy. As an

explanatory hypothesis for the apparent Gbx2 promoter-bound RNA Pol II dynamics we here describe, it may merit future investigation.

Regardless of possible molecular explanations for the above-described modulation of RNA Pol II pausing levels at the Gbx2 promoter, a statistical consideration is here justified. One must consider a possible floor effect as a consequence of the low values being plotted for the Gbx2 promoter Ser5P/Ser2P ratio, especially for the naïve condition, which would result in a set of artificially consistently stable values against which the trained condition would be compared, thus resulting in biologically erroneous statistical significance. Together with biochemical explanations, these statistical aspects should also be considered.

In sum, the analysis of the molecular mechanisms of transcriptional regulation in specific neural circuits will undoubtedly be an exciting research avenue in the future. A further discussion of the areas of intersection between molecular neuroscience and genome biology will be led in the next chapter.

Experimental procedures

Animals. All procedures were reviewed and performed in accordance with the Champalimaud Centre for the Unknown Ethics Committee guidelines, and approved by the Portuguese Veterinary General Board (*Direcção Geral de Veterinária*, approval 0421/000/000/2014). Male C57BL/6J mice between 2 and 5 months of age were used in this study. Experiments were performed on the light cycle.

Behavioural procedures. Behavioural training took place in operant chambers (cm L x cm W x cm H) housed within sound attenuating chambers (MedAssociates, Inc). Each chamber was equipped with two retractable levers on either side of the food magazine and a house light (3W, 24V) mounted on the opposite side of the chamber. Reinforcers were delivered into the magazine through a pellet dispenser, and magazine entries were registered using an infrared beam. Before training started, mice were placed on a food deprivation schedule, receiving 1.5-2g of food per day, allowing them to maintain a body weight above 85% of their baseline weight. Throughout training, mice were fed daily after the training session. Mice were trained with 20mg "chow" pellets (Bio-Serv) as reinforcers, with the delivery of these in the operant chamber contingent upon lever pressing. Training started with a 60 minute magazine training session in which one reinforcer was delivered on a random time schedule on average every two minutes (30 reinforcers). The following day, lever-pressing training started, with each animal learning to press the lever to obtain a reinforcer. Each daily session started with the illumination of the house light and insertion of the lever, and ended with the retraction of the lever and the offset of the house light; sessions lasted for 60 minutes or until animals received a total of 30 reinforcers, with one training session per day. In the first training session, animals were subjected to continuous reinforcement with each lever-press leading to the delivery of one reinforcer into the magazine (to a maximum of 30 reinforcers; CRF30). After CRF, animals were trained in a fixed ratio (FR) schedule, in which delivery of a reinforcer resulted from eight lever-

presses (FR8) within a time contingency, resulting in a minimum frequency (covert target): FR8-1000s (i.e. eight lever-presses within 1000s); FR8-500s; FR8-50s; FR8-10s; FR8-5s; FR8-4s; FR8-3s; FR8-2s; FR8-1s, with animals finishing their fast lever-pressing training at 8Hz. This constant increase in the minimum frequency of the covert target forced the animals to systematically adapt to the task requirements and perform faster sequences of presses from session to session. Seven animals were trained in the fast lever-pressing task, and a control group ("Context control") of seven animals simultaneously exposed to behavioural operant chambers without performing any operant lever-pressing task. An extra control group of animals ("Control w/ pellets", figure 3.4), in which in addition to being exposed to behavioral boxes, animals were fed a maximum of 30 reinforcers per exposure session (similar to experimental subjects upon completion of fast lever-pressing task sessions), was also run. Sequences of lever presses. Sequences of lever presses were differentiated based on inter-press interval (IPI) and occurrence of a magazine head entry. An IPI > 2 seconds (determined based on the distribution of IPIs) or a head-entry were used to define the bouts or sequences of presses.

Western blotting. To dissect whole striata, mice were anesthetized immediately after the termination of behavioral experiments using a mix of oxygen (1–1.5 l/min) and isoflurane (1–3%), sacrificed by cervical dislocation, their brains quickly removed and transferred to ice-cold phosphate buffered saline (PBS). Total striatum was dissected from both hemispheres, flash-frozen in liquid nitrogen and kept at -80ºC until used. Total protein was extracted from the pooled bilateral striata of each mouse by lysis of tissue samples in 400µl of ice-cold RIPA buffer (Sigma-Aldrich, #R0278) supplemented with phosphatase and protease inhibitors (PhosSTOP Roche #04906837001, and Complete Tablets EDTA-free Roche 04693159001, respectively), homogenization using 1.5ml microcentrifuge tube-adaptable disposable tissue grinder pestles (Capitol Scientific, #199230000), disruption by brief sonication and pipetting up and down twenty times with a P200 pipette tip. Protein concentration was assayed using the Pierce BCA Protein Assay

Kit (Thermo Scientific #23227) with the absorbance measured at 562nm on a plate reader, with each animal yielding a protein concentration of 3.000-4.000µg/ml. One part of 4x Laemmli sample buffer (BioRad #161-0747), containing 2-Mercaptoethanol (BioRad #161-0710) in a 1:10 dilution, was added to three parts of protein sample (approximately 40µg of protein per well), boiled at 95ºC for 5 minutes and resolved in 4–15% gradient precast SDS-PAGE gels (Mini-PROTEAN® TGX Stain-Free™ Gels, 10 well, BioRad #456-8083) in 1x running buffer (diluted 1:5 from a 5x stock: 0.125M Tris Base, 1M Glycine, 0.017M SDS), together with a protein ladder for reference (BioRad 1x Precision Plus Protein™ WesternC™ Standards, #161-03764) at 100V for approximately 1.5 hours. Proteins were semi-dry transferred to PVDF membranes (BioRad #162-0177) for 1 hour at 12V in 1x transfer buffer (diluted 1:5 from a 5x stock: 0.125M Tris Base, 0.96M Glycine). PVDF membranes were then blocked in 5% Blotting-Grade Blocker (BioRad #170-6404) in TBS-0.1%Tween20 (TBS: 0.1M Tris, 1.5M NaCl, pH at 7.4) for 1 hour at room temperature (RT). After blocking, PVDF membranes were incubated with the primary antibody at a 1:500 dilution, as well as with an anti-actin antibody (Sigma #A5441) at a 1:200.000 dilution, in TBS-0.1%Tween with 5% Blotting-Grade Blocker over night at 4ºC. Anti-RPB1 primary antibodies used: Total RPB1 subunit — Clone H224 (Santa Cruz Biotechnology #SC-9001X); Ser5P^{+} RPB1 CTD — Clone CTD4H8 (Upstate/Millipore #05-623); Ser2P^{+} RPB1 CTD — Clone H5 (Covance #MMS-129R). After primary antibody incubation, membranes were rinsed three times for 5 minutes with TBS-0.1%Tween at RT, and incubated with the HRP-conjugated secondary antibody at a 1:2000 dilution in TBS-0.1%Tween with 5% Blotting-Grade Blocker for 1 hour at RT. Secondary antibodies used: anti-mouse (Dako #P0260); anti-goat (Invitrogen #G21234). Membranes were then once again washed three times for 5 minutes with TBS-0.1%Tween at RT. The chemiluminescent substrate (Clarity™ Western ECL Substrate, BioRad #170-5060) was added to the blot for 5 minutes at RT according to the manufacturer's recommendations. Chemiluminescent signals were detected in an automated chemiluminescence imager for protein high-resolution digital

imaging (Amersham™ Imager 600). Protein bands were quantified using ImageJ software, with Total RPB1 subunit, Ser5P^{+} RPB1 CTD and Ser2P^{+} RPB1 CTD signals normalized to actin in the respective well.

Chromatin immunoprecipitation (ChIP) followed by RT-qPCR. Similar to Western blotting analysis, mice were anesthetized immediately after the termination of behavioral experiments using a mix of oxygen (1–1.5 l/min) and isoflurane (1–3%), sacrificed by cervical dislocation, their brains quickly removed and transferred to ice-cold phosphate buffered saline (PBS). Total striatum was dissected from both hemispheres, flash-frozen in liquid nitrogen and kept at -80ºC until used. Preparation of Dynabeads Protein G**.** Dynabeads (Life technologies-Invitrogen-Novex 10004D) were mixed well and aliquoted (60 µl per immunoprecipitation reaction), and one tube per antibody prepared. One ml of cold PBS was added to the beads, gently vortexed to mix and the tube placed in a magnetic stand. Tubes were inverted several times to mix, and beads were allowed to clump for approximately 1min. PBS was pipetted off, and this wash step repeated two more times. The specific antibodies were added to the beads: Total RPB1 subunit — Clone H224 (Santa Cruz Biotechnology #SC-9001X) 5 µg/reaction; Anti-RNA polymerase II Ser2P^{+} RPB1 CTD repeat YSPTSPS antibody - ChIP Grade: ab5095, 8µg/reaction; Anti-RNA polymerase II Ser5P^{+} RPB1 CTD repeat YSPTSPS antibody - ChIP Grade: ab5131, 3µg/reaction. The volume was adjusted to 1.5ml with RIPA-150 buffer (50mM Tris-HCl, pH 8.1, 150mM NaCl, 1mM, EDTA pH 8, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate), and antibodies were pre-bound for at least 5 hours at 4ºC on an orbital rotator. While beads were incubated with the antibody, the following crosslinking and lysis steps were performed. *In Vivo* Crosslinking and lysis. 1.5ml tubes were prepared containing 940µl PBS and 60µl fresh formaldehyde (FA) 18.5%, with one tube per mouse bilateral striata. Tissue was chopped using a single-edge razor, transferred into the previously prepared 1.5-ml tube with FA solution and incubated at RT for 10 minutes in an orbital rotator. 110 µl of 1.25 M glycine were then added and

incubated at RT for 5 minutes to quench unreacted formaldehyde. Tubes were spinned at 700G for 3 minutes to pellet tissue and the PBS/FA/glycine solution was aspirated. The tissue was then washed with 1 ml of PBS. The previous 700G spin and 1ml PBS wash cycle was repeated three times, to a total of 3 washes. Next, 500µl of Lysis buffer N (50mM HEPES-KOH pH 8.1, 1mM EDTA, 0.5mM EGTA, 140mM NaCl, 10% Glycerol, 0.5% NP40, 0.25% Triton X-100) with protein inhibitor mixture (Roche #04693159001) were then added to the pellet, and homogenized using a Heidolph Diax 900 homogenizer at level 1 for 10-20 seconds or until no clumps were present in the solution. The homogenate (500µl) was placed into a 15ml tube containing 10ml of Lysis buffer N with protein inhibitor mixture, incubated at 4ºC for 10 minutes with orbital rotation and then spinned at 600G for 5 minutes at 4ºC to pellet nuclei. Nuclei were washed with 10 ml of wash buffer N (10mM Tris-HCl pH 8.0, 1mM EDTA, 0.5mM EGTA, 200mM NaCl) at 4ºC for 10 min with orbital rotation, and pelleted again (600G for 5 minutes at 4ºC). The supernatant was aspirated, and pelleted nuclei resuspended in 100µl of SDS Lysis Buffer (1% SDS, 10mM EDTA, 50mM Tris, pH 8.1). Samples were transferred to 0.5ml LoBind Eppendorf microcentrifuge tubes and sonicated in a Bioruptor (Diagenode) for 20 cycles (30 seconds on / 30 seconds off). Samples were then centrifuged for 6 minutes at 13000 RPM at RT. The pellet (containing insoluble particles) was discarded, and the supernatant (containing sheared chromatin) was transferred to new 1.5 ml LoBind tubes. 5µl (5%, for the total RPB1 subunit experiment) or 10µl (10%, in the Ser2P^{+} and Ser5P^{+} RPB1 experiments) of sheared chromatin were set aside to evaluate shearing efficiency and to measure chromatin concentration (by adding 200µl of freshly made Direct Elution buffer [10mM Tris-HCl pH8, 300mM NaCl, 5mM EDTA pH8, 0.5%SDS] and performing the protein/DNA complex elution and reverse crosslinking to ethanol precipitation steps described below; then dissolving each of the precipitated DNA samples in 20µl of 10 mM Tris-Cl pH8.1, using 5µl to quantify DNA in a Nanodrop system and 15µl to run in a 1.2-1.5% agarose gel [corresponding to 3% of the whole chromatin sample per sample]; DNA fragment size should be in the

range of 200 to 800 bp). Immunoprecipitation of cross-linked protein/DNA. The antibody-bound Dynabeads prepared above were placed in a magnetic stand and inverted several times. Beads were then allowed to clump and the supernatant discarded, with beads being kept on ice. Sonicated chromatin was diluted 1/10 in ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl) with protein inhibitor mixture (the final volume should be 1ml). 1% (10µl) of the supernatant was removed as Input and saved at 4ºC (or -20ºC). Diluted chromatin was added to antibody-bound Dynabeads, gently mixed and placed on a rocker O/N at 4ºC. Tubes were then placed in a magnetic stand and invert several times. Beads were allowed to clump and the supernatant was discarded. The Dynabeads protein G-antibody/chromatin complexes were washed by resuspending the beads in 1ml each of the cold buffers (RIPA-150 buffer for two washes; RIPA-500 buffer [50mM Tris-HCl, pH 8.1, 500mM NaCl, 1mM, EDTA pH 8, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate] for three washes; RIPA LiCl buffer [50mM Tris-HCl, pH 8.1, 1mM EDTA pH 8, 1% NP-40, 0.7%, sodium deosycholate, 500mM LiCl2] for two washes; TE buffer [10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0] for two washes; suds were aspirated after final wash) and incubated for 5 minutes on a rocker at 4ºC. Elution of Protein/DNA complexes and reversal of protein/DNA complex crosslinking. Beads were resuspended in 200µl of freshly made Direct Elution Buffer (with 200µl of freshly made Direct Elution Buffer also added to input samples). From this point on, the protocol was proceeded with proper samples and the saved 1% Input samples. 1µl RNase A 10 mg/ml (Fermentas #EN0531) was added and incubated for 6 hours to O/N at 65ºC to reverse crosslink (samples were kept at 1000 RPM in a termoblock to keep the in suspension). Samples were then quickly spinned and placed on a magnetic stand, allowing beads to clump and transferring supernatants to new LoBind tubes. 3µl of Proteinase K 20mg/ml (Roche #03115879001) were added to each sample and 10µl to each Input and incubated for 1-2 hours at 55ºC. Phenol/chloroform extraction. 2ml phase lock tubes (Fisher #FP2302830) were spinned at RT for 30 seconds at maxG to pellet gel. In the

fume hood, samples were aliquoted into phase lock tubes and an equal volume (approximately 200µl) of phenol/chloroform/isoamyl alcohol was added (Sigma #77617), mixed well and spinned at RT for 5 minutes at maxG. The aqueous phase (aprox 200µl) was transferred into new LoBind 1.5ml tubes. Ethanol precipitation. Two volumes ethanol 100% (aprox. 400µl) were added to the previously prepared aqueous solutions. Then, an additional 8µl 5M NaCl (final concentration 200mM NaCl or 1/10 vol 3M sodium acetate) were added, as well as 1µl glycogen 20ug/ul. The samples were mixed well and frozen at -80ºC for at least 1 hour. Tubes were then spinned in a bench-top microfuge at top speed for 30 minutes at 4ºC, washed with 1ml of cold 70% ethanol solution and spinned again at full speed for 10 minutes at 4ºC. The supernatant was carefully removed and wash step was repeated. The supernatant was removed again and the pellet was dried in a Speedvac**.** DNA was resuspended in 30µl of 10mM Tris-Cl, pH8.1. RT-qPCR. A mix of the adequate PCR primers (5mM each) was prepared. Primers were designed to amplify 50-150bp fragments under very stringent conditions (i.e. Tm 58-60ºC) and were tested both *in silico* and empirically for little or no unspecific amplification. The qPCR mixes were prepared containing: 14µl of H_2O; 4µl of 5x PyroTaq EvaGreen qPCR Mix Plus (CMB Cultek Molecular Bioline #87H24-001); and 1µl of isolated DNA. A plate containing 1µl of primer mix and 19µl of qPCR mix was prepared, and RT-qPCR was performed using an Applied Biosystems 7300 Real-Time PCR System thermocycler with the following protocol: initial denaturation 95ºC for 15 minutes; then 40 cycles of denaturation 95ºC for 15 seconds, annealing 60ºC for 29 seconds and elongation 72ºC for 29 seconds. List of RT-qPCR primers:

GAPDH forward: TTCACCTGGCACTGCACAA;

GAPDH reverse: CCACCATCCGGGTTCCTATAA;

GAPDH gene forward: CTACCCAAAAGGGACACCTACAA;

GAPDH gene reverse: TTTCTTATCTTACCCTGCCATGAG;

Arc promoter forward: GCATAAATAGCCGCTGGTGG;

Arc promoter reverse: GAGAACTCGCTTGAGCTCTGC;

Arc gene forward: TCTCCAGGGTCTCCCTAGTC;
Arc gene reverse: CCCATACTCATTTGGCTGGC;
c-Fos promoter forward: GCAGTCGCGGTTGGAGTAGT;
c-Fos promoter reverse: CGCCCAGTGACGTAGGAAGT;
c-Fos gene forward: GCTTCCCAGAGGAGATGTCTGT;
c-Fos gene reverse: GCAGACCTCCAGTCAAATCCA;
Prkcz promoter forward: GTGGGTCTCCAGATCGACAA;
Prkcz promoter reverse: GCAGGAGAGCCAACCTTCTA;
Prkcz gene forward: CGCCATTGACATACTCGATGA;
Prkcz gene reverse: TCGCCTACAGCATGTTTCG;
Gbx2 promoter forward: CGTGGCAATTTATGGACACAA;
Gbx2 promoter reverse: CACACTTGAAGTAATTTGATGGCAT;
Intergenic region forward: CTACCGAGTGTTGATTGCCGT;
Intergenic region reverse: TGATGCAAGTGTCAAGCTCAATG.

Data analysis. Western blot fold change data (figures 3.2 to 3.4) and ChIP-qPCR % of Input data (figures 3.5 to 3.8) were generated from 7 animals per group (naïve or trained) for Western blot analysis (with 3-5 replicate wells in independent gel runs per animal); and a minimum of 3 animals per group (naïve or trained) for ChIP-qPCR analysis (with a minimum of two replicate C_T measurement repeats per qPCR experiment). Data was expressed as mean ± SEM, and statistically evaluated at a significance level of 5% with unpaired Student's *t* test (*, $P < 0.05$) (comparing naïve to trained groups for the Western blot analysis; or naïve to trained groups, and Ser5 to Ser2 levels, for the ChIP-qPCR analysis for each individual target [i.e. promoter or gene body]), using GraphPad Prism® (GraphPad Software). Results were represented as mean ± SEM. For behavioural analysis, a one-way ANOVA was used to evaluate acquisition of inter-press intervals and distances to target. Statistical significance was set at alpha=0.05. Figure symbols are as follows: *, $P < 0.05$, **, $P < 0.01$ ***, $P < 0.005$; n.s., $P > 0.05$.

References

Aoki S, Liu AW, Zucca A, Zucca S and Wickens JR (2015) **Role of Strital Cholinergic Interneurons in Set-Shifting in the Rat**. Journal of Neuroscience 35: 9424-9431.

Chen L, Chatterjee M and Li JY (2010) **The mouse homeobox gene Gbx2 is required for the development of cholinergic interneurons in the striatum**. Journal of Neuroscience 44: 14824-14834.

Gilmour DS and Lis JT (1986) **RNA polymerase II interacts with the promoter region of the non-induced *hsp70* gene in *Drosophila melanogaster* cells**. Molecular and Cellular Biology 6: 3984-3989.

Hager GL, McNally JG and Misteli T (2009) **Transcription dynamics**. Molecular Cell 35: 741-753.

Islam ME, Kikuta H, Inoue F, Kanai M, Kawakami A, Parvin MS, Takeda H and Yamasu K (2006) **Three enhancer regions regulate gbx2 gene expression in the isthmic region during zebrafish development**. Mechanisms of Development 123: 907-924.

Jonkers I and Lis JT (2015) **Getting up to speed with transcription elongation by RNA polymerase II**. Nature Reviews Molecular Cell Biology 16: 167-177.

Lenhard B, Sandelin A and Carninci P (2012) **Metazoan promoters: emerging characteristics and insights into transcriptional regulation**. Nature Reviews Genetics 13: 233-245.

Levine M, Cattoglio C and Tjian R (2014) **Looping back to leap forward: transcription enters a new era**. Cell 157: 13-25.

Lim SAO, Kang UJ and McGehee DS (2014) **Striatal cholinergic interneuron regulation and circuit effects**. Frontiers in Synaptic Neuroscience doi: 10.3389/fnsyn.2014.00022.

Lyons MR and West AE (2011) **Mechanisms of specificity in neuronal activity-regulated gene transcription**. Progress in Neurobiology 94: 259-295.

Meaney MJ and Ferguson-Smith AC (2010) **Epigenetic regulation of the neural transcriptomes: the meaning of the marks**. Nature Neuroscience 13: 1313-1318.

Okada K, Nishizawa K, Fukabori R, Kai N, Shiota A, Ueda M, Tsutsui Y, Sakata S, Matsushita N and Kobayashi K (2014) **Enhanced flexibility of place discrimination learning by targeting striatal cholinergic interneurons**. Nature Communications 5 doi: 10.1038/ncomms4778.

Pérez-Cadahía B, Drobic B and Davie JR (2011) **Activation and function of immediate-early genes in the nervous system**. Biochemistry and Cell Biology 89: 61-73.

Rasmussen EB and Lis JT (1993) **In vivo transcriptional pausing and cap formation on three *Drosophila* heat shock genes**. Proceedings of the National Academy of Sciences USA 90: 7923-7927.

Rougvie AE and Lis JT (1988) **The RNA Polymerase II molecule at the 5' end of the uninduced *hsp70* gene of *D. melanogaster* is transcriptionally engaged**. Cell 54: 795-804.

Rougvie AE and Lis JT (1990) **Postinitiation transcriptional control in *Drosophila melanogaster***. Molecular and Cellular Biology 10: 6041-6045.

Sacktor TC, Osten P, Valsamis H, Jiang X, Naik MU and Sublette E (1993) **Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation**. Proceedings of the National Academy of Sciences USA 90: 8342:8346.

Saha RN, Wissink EM, Bailey ER, Zhao M, Fargo DC, Hwang JY, Daigle KR, Fenn JD, Adelman K and Dudek SM (2011) **Rapid activity-induced transcription of *Arc* and** other IEGs relies on poised RNA Polymerase II. Nature Neuroscience 14: 849-856.

Stock JK, Giadrossi S, Casanova M, Brookes E, Vidal M, Koseki H, Brockdorff N, Fisher AG and Pombo A (2007) **Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells**. Nature Cell Biology 9: 1428-1435.

Wassarman KM, Lewandoski M, Campbell K, Joyner AL, Rubenstein JL, Martinez S and Martin GR (1997) **Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function**. Development 124: 2923-2934.

West AE and Greenberg ME (2011) **Neuronal activity-regulated gene transcription in synapse development and cognitive function**. Cold Spring Harbor Perspectives in Biology 3: 1-21.

Wolf C and Linden DEJ (2012) **Biological pathways to adaptability — interactions between genome, epigenome, nervous system and environment for adaptive behavior**. Genes, Brain & Behavior 11: 3-28.

"If you know you are on the right track, if you have this inner knowledge,
then nobody can turn you off... no matter what they say."

Barbara McClintock

Chapter IV

General discussion:

From striatal circuit function to RNA Pol II pausing

When the research work for this thesis began, its primary intention was to fully bridge the gap between circuit neurobiology and the myriad of epigenetic mechanisms known to operate in neurons. The molecular pathways that allow chromatin to dynamically restructure itself and the transcription machinery to adapt to divergent extracellular and intracellular signals haven't typically been described, in the brain, with cell-type specificity, but rather at the level of brain structures or areas (e.g. the hippocampus, cortex or striatum). Thus, according to our initial experimental design, a starting point of optogenetic circuit-level manipulation — with its own conceptual agenda: that of unraveling controversial functional details within the two main output pathways of the striatum — would evolve to a circuit-specific examination of the biochemistry of neuronal genomes as these responded to precisely-timed and quasi-physiological patterns of activity. The work of Saha et al. (2011) had already proven that the mechanism known as RNA Pol II pausing is present in the central nervous system, is regulated by neuronal activity and is responsible for the fast induction kinetics of IEGs. With the purification of optogenetically-controlled neural circuits (i.e. a population of genetically-identified neurons, onto which we imposed a defined pattern of neural activity), we intended to study the genome kinetics of RNA Pol II pausing in the mouse brain with cell-type specificity.

With this conceptual and experimental framework in mind, we began by examining the differential roles played in positive reinforcement by the two main striatal output pathways (the direct, striatonigral, dopamine D1 receptor-expressing pathway and the indirect, striatopallidal, dopamine D2 receptor-expressing striatal pathway). According to recent work, the two striatal output pathways were assigned opposing roles in action reinforcement, with the striatonigral pathway supporting positive reinforcement, while the striatopallidal pathway coded for action avoidance (Kravitz et al., 2012). Here, using a self-stimulation optogenetics instrumental task, we show that self-stimulation of either pathway in dorsolateral striatum leads to positive reinforcement, but that this stimulation supports the development of different action strategies. More specifically, we demonstrate that activation of

striatonigral neurons resulted in rapid and robust action-specific reinforcement, while stimulation of striatopallidal neurons led to generalization to similar actions and a decreased sensitivity to action-stimulation contingency.

To support our finding that self-stimulation of both striatonigral and striatopallidal DLS neurons is sufficient to positively reinforce actions, but that stimulation of each pathway supports the learning of different action strategies, previous work from our group has shown that in DLS both the striatonigral and striatopallidal pathways are active during lever pressing to receive a reward (Jin et al., 2014). These results suggest that pairing activation of striatonigral neurons in DLS with an action leads to the establishment of a goal-directed action, while pairing activation of striatopallidal neurons in DLS with an action supports the formation of a habit that is insensitive to changes in contingency. These results support the idea that DLS might not be an overall homogenous structure involved in habit formation, but that there is a different involvement of the direct and the indirect pathways in this area; during habit formation, potentiation of indirect MSNs in DLS could lead to the formation of a habit, possibly due to competition/inhibition between the two pathways. These results fit neatly with the previously described role of DLS striatopallidal neurons in skill consolidation — where extensive training was shown to lead to long-lasting potentiation of glutamatergic inputs into both striatonigral and striatopallidal DLS neurons — and habit formation — where striatal-specific deletion of A_{2A} receptors abolishes long-term potentiation onto striatopallidal neurons, impairing the development of habitual actions (Yin et al., 2009; Yu et al., 2009). The DMS might play a divergent role in reinforcement, where the direct and indirect pathways have been shown to support different and opposing roles in reinforcement (Kravitz et al., 2012). Both striatal output pathways may, of course, be involved in action selection, with striatonigral neurons supporting the execution of the desired action pattern, and striatopallidal neurons avoiding the execution of competing action patterns (Mink, 1996; Cui et al., 2013). Although our results differ from those in DMS, there are several experimental differences between these studies. Besides the stimulation sites

and stimulation patterns, the reinforced actions are different for both studies. Thus, it is not possible to conclude, necessarily, that the observed discrepancies are due to different roles of striatonigral and striatopallidal neurons in both regions. One single, major conclusion, however, remains: that in DLS, self-stimulation of striatopallidal neurons is not aversive.

With these data, we provide a contribution to an increasingly widespread model of basal ganglia function, according to which the direct and indirect striatal output pathways are not simply dichotomic, but work alongside each other to regulate action performance (Mink, 1996; Gerfen and Young, 1988; Albin et al., 1989; Hikosaka, 2000; for a review of more classical views of basal ganglia function: Kreitzer and Malenka, 2008; Graybiel and Grafton, 2015).

According to our initial plan, we proceeded to attempt the purification of optogenetically-controlled neurons for transcriptome analysis. We performed FACS on striatal tissue dissected from optogenetically-controlled D1- and D2-YFP/ChR2-YFP mice and extracted RNA from FACS-purified cells. We were, however, unable to extract RNA of enough quality and quantity from the purified cell populations to warrant further transcriptomics approaches (such as RNA-Seq).

Alongside other mechanisms of activity dependent gene expression, the regulation of RNA Pol II progression from gene promoters to downstream coding regions is now seen as a major player in transcriptional regulation (reviewed in Brookes and Pombo, 2009; Heidemann et al., 2013; Jonkers and Lis; 2015). The serine phosphorylation events along the CTD of RPB1 (which comprises approximately fifty-two repeats of the heptapeptide $YS_2PTS_5PS_7$) code the relative position of RNA Pol II molecules: the most $Ser5P^+$-enriched RPB1 mainly present in promoter-proximal regions, and $Ser2P^+$-enriched RPB1 in actively transcribing RNA Pol II molecules (Brookes and Pombo, 2009; Heidemann et al., 2013; Jonkers and Lis; 2015). RNA Pol II pausing has been shown to be modulated in neuronal cortical cultures (in which the onset of neural activity, brought on by tetrodotoxin withdrawal, promotes the transition of RNA Pol II from a promoter-bound to an actively transcribing state), where it is, at least partly, responsible for the fast

expression kinetics of IEGs such as Arc or c-fos (Saha et al., 2011). However, this mechanism has never been shown to be modulated by high-level processes such as learning (which have, for a long time, been known to involve chromatin remodeling and other instances of transcriptional regulation; reviewed in West and Greenberg, 2011; Wolf and Linden, 2012). Here, we decided to ask whether learning a motor skill would modulate the dynamics of RNA Pol II phosphorylation in the mouse striatum. We taught mice an operant motor task in which they had to press a lever at up to 8Hz in order to receive a food reward, and probed their striatal proteins for total RPB1 CTD, Ser5P^{+}- and Ser2P^{+}-enriched RPB1. We show that learning modulates the *in vivo* striatal phosphorylation dynamics of RNA Pol II RPB1. We demonstrate that a decrease of striatal levels of Ser2P^{+}-enriched RPB1 is present in the striata of trained mice when compared to controls that did not learn the motor skill (while levels of Ser5P^{+}-enriched RPB1 CTD between naïve and trained mice remained constant). This decrease in Ser2P^{+} RPB1 results in an increased pausing index for trained mice when compared to their naïve littermates. We also show that RNA Pol II RPB1 phosphorylation is modulated for the Arc and c-Fos IEGs, both of which demonstrate a slight increase in Ser5P^{+}-enriched RPB1-binding and decrease in Ser2P^{+}-enriched RPB1 binding, together with a higher pausing index of IEG-bound RNA Pol II. Taken together, these experiments provide the first demonstration of RNA Pol II phosphorylation modulation in the adult brain in the context of learning.

These results fit with the molecular scenario described by Saha et al. (2011), who showed neuronal activity-regulated changes in RNA Pol II pausing at rapidly-induced IEGs, such as Arc and c-Fos (the same targets examined in our experiments). Coherently, we found an increase in the pausing indexes of IEG-bound RNA Pol II when mice were subjected to a motor learning paradigm. These molecular dynamics may result in a wider re-organization at the level of gene promoters to facilitate activity-induced fast transcription (mechanisms reviewed in Flavell and Greenberg, 2008; Saha and Dudek, 2013). A very recent study unearthed the importance of c-Fos enhancers on regulating its activity-dependent

induction, advancing our understanding of how different stimuli result in the combinatorial activation of various enhancers, beyond the well-described c-Fos promoter (Joo et al., 2016). These new and exciting results bring activity-dependent gene expression closer to a thorough examination of neuronal nuclear dynamics.

The work from the Dudek lab, profusely alluded to throughout this thesis, has already demonstrated that RNA Pol II pausing results in a kinetic advantage to the transcription of rapidly-induced IEGs, including Arc and c-fos (Saha et al., 2011). They also demonstrate that neuronal activity promotes the phosphorylation of the RPB1 CTD at Ser2, releasing RNA Pol II molecules from promoters, inducing their transition to active elongation. We observe a similar process in the mouse striatum as a response to skill learning. Learning may thus be accompanied by a modulation of pausing indexes, whereby an increase in Ser5P^{+}-rich RNA Pol II promoter-bound molecules could result in the consolidation of learning by adapting gene responses to neuronal activity (Saha and Dudek, 2013). Our results inscribe a new chapter within the solid research tradition on neuronal activity-dependent gene expression, and contribute to an understanding of how differently transcriptional modulation occurs in different brain systems and circuits.

Recent progress has been made on capturing RNA Pol II pausing dynamics as it transcribes RNA from mammalian genomes. These new and improved sequencing techniques take us one step closer to a nucleotide-resolution examination of RNA Pol II occupancy, and have already added some interesting insights into the known relationship between RNA Pol II site-specific pausing and its binding to exon boundaries and tuning of nascent transcript splicing (Mayer et al., 2015; Nojima et al., 2015). These new studies have only further highlighted the functional complexity of the RPB1 CTD combinatorial code. It will be undoubtedly interesting to apply these new methodologies to neural circuits and activity-dependent alternative splicing.

One thing, though, is certain: as methods for circuit-specific molecular analysis are perfected, and insights into the molecular epigenetic details of neuronal functioning increase, the study of neural circuit-specific transcriptional regulation

will, without a doubt, be at the forefront of molecular neurobiological research in the future.

As a testimony to this trend, Figure 4.1 presents the structural dynamics of two activity-dependent transcriptional units, adapted from review articles in completely different fields of biomedical specialization: one specifically neuronal in nature, the other more general. Notwithstanding the aesthetic similarities, both schemes present an already convergent vision of gene expression regulation that incorporates several dimensions of spatial complexity, from the molecules involved, to their 3-D geography.

Much research has recently been devoted to unraveling the mechanisms behind the cell type-specific regulation of gene expression within its nuclear context. Topologically associating domains (TADs) and chromosome boundaries, held together and regulated by cohesin and the CCCTC-binding factor (CTCF), orchestrate the hierarchically complex chromatin organization of mammalian nuclei. The local phenomena responsible for enhancer-promoter interaction-mediated chromatin looping are only now being tackled in detail (Kieffer-Kwon et al., 2013; Chen et al., 2014; Crosetto et al., 2015; Schwartzman and Tanay, 2015). The tracking of epigenetic mechanisms in single cells on a genome-wide scale promises to examine the very small and very specific molecular context of the individual cell within the larger reality of the tissue, linking molecular systems to systems molecular biology (Schwartzman and Tanay, 2015).

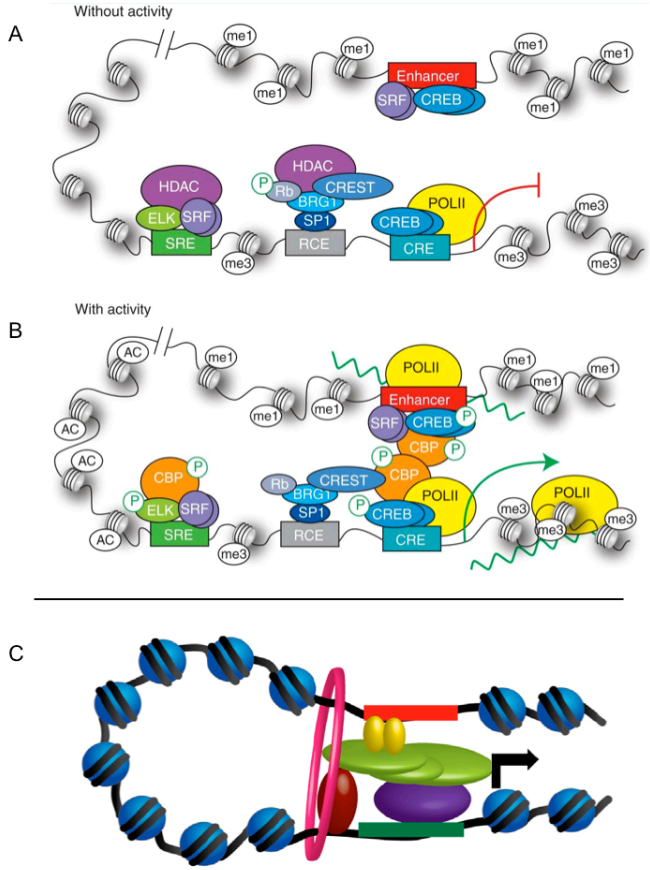


Figure 4.1: **Transcription of prototypical activity-dependent genes.** Panels A and B (adapted from West and Greenberg, 2011) represent the neuronal activity-dependent transcription of *Fos*, an IEG; while panel C (adapted from Gross et al., 2015) depicts chromatin looping resulting from promoter-enhancer interactions (the loop is stabilized by cohesin, here represented as a pink ring, the enhancer and promoter are represented by red and green rectangles, respectively, RNA Pol II in purple, Mediator in green and CTCF in orange).

As the bridge between external reality and internal biology, the brain and its study by neuroscience are natural inheritors of Conrad Waddington's visionary spirit. His epigenetic landscape is a brilliant conceptual roadmap to the genome's functional complexity.

Neuroscience is jumping on the dynamic regulatory genome train.

References

Albin RL, Young AB and Penney JB (1989) **The functional anatomy of basal ganglia disorders**. Trends in Neuroscience 12: 366-375.

Brookes E and Pombo A (2009) **Modifications of RNA Polymerase II are pivotal in regulating gene expression states**. EMBO Reports 10: 1213-1219.

Chen J, Zhang Z, Li L, Chen BC, Revyakin A, Hajj B, Legant W, Dahan M, Lionnet T, Betzig E, Tjian R and Liu Z (2014) **Single-molecule dynamics of enhanceosome assembly in embryonic stem cells**. Cell: 156: 1274-1285.

Crosetto N, Bienko M and van Oudenaarden A (2015) **Spatially resolved transcriptomics and beyond**. Nature Reviews Genetics 16: 57-66.

Cui G, Jun SB, Jin X, Pham MD, Vogel SS, Lovinger DM and Costa RM (2013) **Concurrent activation of striatal direct and indirect pathways during action initiation**. Nature 494: 238-242.

Flavell SW and Greenberg ME (2008) **Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system**. Annual Review of Neuroscience 31: 563-590.

Gerfen CR and Young WS III (1988) **Distribution of striatonigral and striatopallidal peptidergic neurons in both patch and matrix compartments: an in situ hybridization histochemistry and fluorescent retrograde tracing study**. Brain Research 460: 161-167.

Graybiel AM and Grafton ST (2015) **The striatum: where skills and habits meet**. Cold Spring Harbor Perspectives in Biology 7:a021691.

Gross DS, Chowdhary S, Anandhakumar J and Kainth AS (2015) **Chromatin**. Current Biology 25: 1158-1163.

Heidemann M, Hintermain C, Voβ K and Eick D (2013) **Dynamic phosphorylation patterns of RNA Polymerase II CTD during transcription**. Biochimica aet Biophysica Acta 1829: 55-62.

Hikosaka O., Takikawa Y., Kawagoe R. (2000). **Role of the basal ganglia in the control of purposive saccadic eye movements**. Physiological Reviews 80, 953–978

Jin X, Tecuapetla F and Costa RM (2014) **Basal ganglia subcircuits distinctively encode the parsing and concatenation of action sequences**. Nature Neuroscience 17: 423-430.

Joo JY, Schaukowitch K, Farbiak L, Kilaru G and Kim TK (2016) **Stimulus-specific combinatorial functionality of neuronal *c-fos* enhancers**. Nature Neuroscience 19: 75-83.

Jonkers I and Lis JT (2015) **Getting up to speed with transcription elongation by RNA polymerase II**. Nature Reviews Molecular Cell Biology 16: 167-177.

Kieffer-Kwon KR, Tang Z, Mathe E, Qian J, Sung MH, Li G, Resch W, Baek S, Pruett N, Grøntved L, Vian L, Nelson S, Zare H, Hakim O, Reyon D, Yamane A, Nakahashi H, Kovalchuk AL, Zou J, Joung JK, Sartorelli V, Wei CL, Ruan X, Hager GL, Ruan Y and Casellas R (2013) **Interactome maps of mouse gene regulatory domains reveal basic principles of transcriptional regulation**. Cell 155: 1507-1520.

Kravitz AV, Tye LD and Kreitzer AC (2012) **Distinct roles for direct and indirect pathway striatal neurons in reinforcement**. Nature Neuroscience 15: 816-818

Kreitzer AC and Malenka RC (2008) **Striatal plasticity and basal ganglia circuit function**. Neuron 60: 543-554

Mayer A, di Iulio J, Maleri S, Eser U, Vierstra J, Reynolds A, Sandstrom R, Stamatoyannopoulos JA and Churchman LS (2015) **Native elongating transcript**

sequencing reveals human transcriptional activity at nucleotide resolution. Cell 161: 541-554.

Mink JW (1996) **The basal ganglia: focused selection and inhibition of competing motor programs**. Progress in Neurobiology 50: 381-425.

Nojima T, Gomes T, Grosso AR, Kimura H, Dye MJ, Dhir S, Carmo-Fonseca M, Proudfoot NJ (2015) **Mammalian NET-Seq Reveals Genome-wide Nascent Transcription Coupled to RNA Processing**. Cell 161: 526-540.

Saha RN, Wissink EM, Bailey ER, Zhao M, Fargo DC, Hwang JY, Daigle KR, Fenn JD, Adelman K and Dudek SM (2011) **Rapid activity-induced transcription of *Arc* and other IEGs relies on poised RNA Polymerase II**. Nature Neuroscience 14: 849-856.

Saha RN and Dudek SM (2013) **Splitting hares and tortoises: a classification of neuronal immediate early gene transcription based on poised RNA Polymerase II**. Neuroscience 247: 175-181.

Schwartzman O and Tanay A (2015) **Single-cell epigenomics: techniques and emerging applications**. Nature Reviews Genetics 16: 716-726.

West AE and Greenberg ME (2011) **Neuronal activity-regulated gene transcription in synapse development and cognitive function**. Cold Spring Harbor Perspectives in Biology 3: 1-21.

Wolf C and Linden DEJ (2012) **Biological pathways to adaptability — interactions between genome, epigenome, nervous system and environment for adaptive behavior**. Genes, Brain & Behavior 11: 3-28.

Yin HH, Mulcare SP, Hilário MRF, Clouse E, Holloway T, Davis MI, Hansson AC, Lovinger DM and Costa RM (2009) **Dynamic reorganization of striatal circuits during the acquisition and consolidation of a skill**. Nature Neuroscience 12: 333-341.

Yu C, Gupta J, Chen J and Yin HH (2009) **Genetic deletion of A_{2A} adenosine receptors in the striatum selectively impairs habit formation**. Journal of Neuroscience 29: 15100-15103.

ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

www.itqb.unl.pt